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# The effect of ethylenethiourea on the developing central nervous system of the rat

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**The effect of ethylenethiourea on the developing  
central nervous system of the rat**

**by**

**Pisut Mungkornkarn**

**A Dissertation Submitted to the  
Graduate Faculty in Partial Fulfillment of  
The Requirements for the Degree of  
DOCTOR OF PHILOSOPHY**

**Department: Veterinary Anatomy,  
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## TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	5
Ethylenethiourea	5
The development of the CNS in the Rat	8
Nutritional Factors	
Hypovitaminosis and hypervitaminosis A	11
Riboflavine deficiency	12
Folic acid deficiency	13
Niacin deficiency	14
Pantothenic acid deficiency	15
Vitamin E deficiency	15
Purine and pyrimidine antimetabolites	16
Metals	17
Physical Factors	19
Radiation	19
Stress	22
Hypoxia	22
Genetic Factor	23
Hormonal Imbalances	24
Drug	24
Infectious Agents	25
MATERIALS AND METHODS	27
OBSERVATION	37
Microscopic Observation	48
Exencephaly	48
Hydrocephalus	49
Hydrocephalus with exencephaly	52
Hydranencephaly	54

	Page
DISCUSSION	56
SUMMARY	65
BIBLIOGRAPHY	67
ACKNOWLEDGMENTS	79
APPENDIX	80

## INTRODUCTION.

The central nervous system develops from the embryonic ectoderm, which forms the neural plate under the inductive influence of the notochord; later the neural plate develops into neural folds. Neural tube is the result of fusion of neural folds. Defective closure of the neural tube during the early developing process of the central nervous system could result in congenital malformations. The defects may be primarily of the nervous system itself but may also affect the other systems. Congenital malformations of the central nervous system are common in both humans (Emanuel et al., 1972; Hidaka et al., 1974) and animals (Kalter, 1968).

Malformations can be caused by genetic abnormalities, environmental factors, or the interaction of both. The causes of developmental defects in man have been estimated by Wilson (1973). The contribution of known genetic transmission and chromosomal aberrations is about 25 percent, and the estimate of environmental cause does not exceed 10 percent toward the generation of anomalous development. The rest of the percentage seems to be significant for the unknown causation, which could be the effect of combination of both genetic and environmental factors on the developing organisms. In terms of the environment, any one of the multiple factors could influence the developing embryos or fetuses--such as the amniotic fluid, the enclosing fetal membranes, uterus, maternal body, infection, physical and chemical agents, and others. Presently, there is nothing much to reduce the genetic predisposition. Therefore, every effort has been concentrated on the environmental factors. It was believed that

congenital malformations might result by exposure to the environment during pregnancy (Janerich, 1971; Emanuel and Sever, 1973). The mechanism of malformation is still not clearly understood. Tazima (1975) stated that the internal response of a developing system to an environmental cause must be translated into either the morphogenetic or biochemical events that will lead finally to structural and functional abnormalities, prenatal death, or growth retardation.

Epidemiology of malformations has indicated that there is a large incidence of abnormalities in the central nervous system, especially anencephaly and spina bifida (ASB), due to environmental cause (Macmahon and Yen, 1971). Morton and Elwood (1974) found a significant correlation between the neural tube defects and the presence of aluminum trace element in the hard water in South Wales. Renwick (1973), on the other hand, mentioned the seasonal peak incidence between poor potato quality in May or June and the high number of ASB in late April. As indicated by Leck (1972), the genotype plays a small role in the neural tube defects but the environmental factors, season, maternal age, parity, and socioeconomic status are more important in his view.

Since the thalidomide catastrophe of 1961, much interest in developmental malformations of the mammalian fetuses has been stimulated. Many of the drugs used during pregnancy or accidentally have been investigated in the laboratory with the experimental animals to test their teratogenicity. Unlike drugs, another group of chemicals used in the spraying of agricultural plants such as fungicides, herbicides, and insecticides have also caused concern as poisonous pollutants in the environment. They



have been used for pest control for a considerable period and the older ones, such as arsenic, nicotine, and sulphur, appear to be more hazardous. Some of the pesticides have been shown to be teratogenic.

In the agricultural world, soil, water, and plants have been treated with a considerable amount of pesticides, herbicides, and fungicides, which are used for the control of diseases and are applied as a spray, dust, or in a granular form. If care is not taken during the application of these chemicals, they will affect human health adversely. Little is known about the degradation, action or toxicity of these pesticides after their application. Without the metabolism of the organism, it is believed that certain oxidative and hydrolytic processes may accelerate the degradation of these chemical compounds. A number of toxic organic compounds are unstable in water and may be broken down into inactive substances. Many residues of some of these substances are quite persistent for 20 years. High mortality of wild animals and birds has been evident as a result of toxicity of these groups of chemicals in the carcasses. This effect rendered them unsafe for human consumption.

Weeds and fungal infections can lower the agricultural yield of plants and decrease food production. Fungal diseases of plants are difficult to control chemically because of their intimate relationship with the host-parasite. Chemical agents can cause many different types of injury, such as skin irritation and malformation of newborn organisms; they may damage the genetic structure in either somatic or reproductive cells. Organomercury, which is more toxic than other fungicides, was one of the first groups used as a weed dressing and wood preservative. Toxicity of

alkylmercury affected the central nervous system, resulting in poor muscular coordination.

After World War II many organic fungicides such as Thiram (Tetramethylthiuramdisulphide) and dithiocarbamate were developed. Some toxicity and teratogenicity of ethylenebisdithiocarbamate (EBDC) had been studied in lower forms of animals and in mammals as well. It was demonstrated that the teratogenicity of these organic compounds was due to their common metabolite, alkylurea. Although experiments have been conducted about the teratogenicity of alkylthiourea and ethylenethiourea (breakdown products of EBDC fungicide), details of their effects on the central nervous system still remain to be investigated. Ethylenethiourea given to pregnant rats on day 12 of gestation at dose levels of 300 mg/kg per os produced a variety of defects on the central nervous system of the fetal rats in our screening experiment. Therefore, the present studies were initiated to study the effects of ethylenethiourea on the nervous system of the fetal rats.

## REVIEW OF LITERATURE

## Ethylenethiourea

Ethylenethiourea (ETU) is one of the decomposition compounds of the ethylenebisdithiocarbamate group of fungicides (Newsome and Lever, 1973; Tweedy, 1973). It has been identified in more than 20 commercial products of the fungicides (Bontoyan and Looker, 1972). ETU can be synthesized from ethylenediamine and carbondisulfide in the presence of hydrochloric acid. The melting point of ETU is 196°C (Allen et al., 1946). The chemical purity can also be obtained by recrystallizing from 2-imidazolidinethione<sup>1</sup> and determined by thin layer chromatography (Ruddick and Khera, 1975). ETU is soluble in water to the extent of 2 gm/liter at 30°C (Strecher, 1968). It is very slightly soluble in common organic solvent, except in the low concentration of methanol and ethanol (Ross and Crossby, 1973). The aqueous solution of ETU is very stable towards hydrolysis over the pH range from 5.0 to 9.0 but decomposition of the solution occurs rapidly in the presence of photosensitizers, such as, methylene blue and eosin (Cruickshank and Jarrow, 1973). Ross and Crossby (1973) indicated that natural photosensitizer is important to ETU decomposition in boiled sample of agricultural drainage water. The pathway of ETU degradation is not know.

The commercial ethylenebisdithiocarbamate (EBDC) formulation kept in the garage is likely to degrade to ETU, ethylenethiurammonosulfide, sulfur, and metal sulfide (Bonotoyan and Looker, 1972). Petrosini et al.

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<sup>1</sup>Eastman-Kodak, Rochester, New York.

(1963) identified ETU, carbondisulfide and zinc sulfide from the commercial products, Zineb (Zinc-ethylenebisdithiocarbamate) and Ziram (Zinc-dimethyldithiocarbamate) under different storage conditions. The mechanism of EBDC degradation to ETU is still unknown (Newsome and Lever, 1973). Boiling of foods containing the original compound ethylenebisthiocarbamate increases the ETU to significant levels (Blazquez, 1973; Newsome and Lever, 1973; Watts et al., 1974). Used to identify the ETU residues in plants are several techniques such as thin-layer and gas liquid chromatography, infrared spectrophotography, and mass spectrography (Bontoyan and Looker, 1972).

Fate of ETU and EBDC has been studied in both plants and animals. After application of  $C^{14}$  labelled ETU and EBDC to the soil growing soybean, the radioactivity found in the leaves and the labelled ETU content in the leaves frozen for several months was higher than freshly harvested leaves (Nash, 1975). Most of the metabolic fate of ETU in both the rat and mouse is still unknown. Ruddick et al. (1976a) found that ETU was metabolized to ethyleneurea, and one unidentified product. ETU was excreted rapidly in the urine of the rat and guinea pig and about 50 percent of administered dose was excreted without change in 24 hours, whereas the concentration of ETU was increased markedly in the thyroid glands of both species (Newsome, 1974). Ruddick et al. (1976b) studied the distribution, excretion, and metabolism of ETU in the pregnant rat. Following administration of a single oral dose of  $C^{14}$  labelled ETU for 2 hours, the level of radioactivity was distributed evenly in some maternal tissues,

higher than in the embryos. About 72 percent of the administered dose had been excreted in the urine.

Recent reports have indicated that ETU is a thyroid carcinogenic agent (Innes et al., 1969; Ulland et al., 1972; Graham et al., 1973). The effect of long-term feeding for one year, with lower dose of 135 ppm (Graham et al., 1973), or short term feeding with higher dose of less than 500 ppm (Graham and Hansen, 1972) induced hyperplasia of the thyroid. ETU was also found to be tumorigenic in mice (Innes et al., 1969).

Several experiments have shown ETU to be teratogenic in rat maternally at subtoxic levels. The acute LD<sub>50</sub> (half lethal dose) for ETU in rat is 1832 mg/kg with confidence interval from 1379-2562 mg/kg (Graham and Hansen, 1972). Single oral administration (Ruddick and Khera, 1975; Teramoto et al., 1975) on day 10 to 21 of pregnancy or multiple administration on day 6 to 15 of pregnancy (Khera, 1973) produced high rates of visceral and skeletal anomalies in rats. Bal and Khera (1975) reported undescended or partially descended testes, and disconnection of Müllerian duct with the urogenital sinus and other associated abnormalities in the rat dosed orally with ETU (480 mg/kg). No teratogenicity was found in the rabbit (Khera, 1973) and mice (Ruddick et al., 1976a). Postnatal study in the rat revealed that ETU induced hydranencephaly (Khera and Tryphonas, 1976).

ETU was also reported to be mutagenic. Base substitution in the DNA of Salmonella typhimurium occurs without affecting the RNA and protein synthesis (Seiler, 1974). It affects xy-bearing sperm in a different way, resulting in absence of sex chromosome (Mollet, 1975). The carcinogenic

or mutagenic properties of ETU may be increased if sodium nitrate was given at the same time (Seiler, 1975).

### The Development of the CNS in the Rat

The early development of the central nervous system of the albino rat, Mus norvegicus albinis, from day 1 to 9, was fully described by Huber (1915). The study was conducted from fertilization up to the early developmental stage of the mesoderm and the anlage of the primitive streak. Adelmann (1925) has studied the later developmental part, which primarily concerned the development of the nervous system up to the 26-somite stage. He described the formation of the neural folds from the neural plate on day 10. These neural folds began to elevate in a first somite embryo. There was a marked expansion when the neural folds started to diverge in a 2-8 somite embryo. During the 5-8 somite stage the head region grew enormously while the lateral borders of the neural plate in this region began to move medially initiating the closure of the neural tube. Karfunkel (1974) proposed the mechanism of neural tube formation, which involved some forces residing in the neural ectoderm. The microtubules affected cell elongation, whereas the microfilaments influenced apical constriction of cells.

Neural tube consists primarily of three zones: germinal epithelial, mantle, and outer zone (Langman et al., 1966). The germinal neuroepithelial (Hicks, 1958), or the primitive ependymal cells (Sidman et al., 1959), or matrix cell layer (Fujita, 1963) are pseudostratified columnar epithelium. With tritiated thymidine and autoradiographic technique, this

layer was observed to consist of homogeneous cell population (Fujita, 1963), which was previously believed to be consisting of two types of cells, germinal cells and spongioblasts (Saver, 1935). The mechanisms of proliferation and migration of the neuroepithelial cells in the neural tube has been studied (Sidman et al., 1959; Fujita, 1963; Langman et al., 1966). During DNA synthesis the nuclei of the undifferentiated epithelial cells are in the external half of the matrix cell layer. To undergo mitosis these nuclei then migrate towards the internal limiting membrane of the ventricle. Most of the mitotic figures have spindle fibres arranged parallel to the ventricular surface. After cell division nuclei of these cells migrate laterally and some participate in formation of the mantle layer, or they remain in the primitive ependymal zone and repeat the cycle.

In a 9-somite embryo, 10 1/2 days old, the neural canal is closed from the level of second to the sixth somite. The rostral limits of the hind brain and five rhombomeres are formed at this time. Rhombomeres were regarded by Adelmann (1925) as an expression of growth factors in a limited space. Between the 10th to 14th embryonic somites the prosencephalon differentiates into telencephalon and diencephalon. Segmentation of the dimesencephalic region disappears and the neural folds fuse at the diencephalic-mesencephalic region (Christie, 1964).

The central hemispheres form at the 16-somite stage (11 days of development) and become more prominent at 14-15 days (Long and Burlingame, 1938). In the 18-somite embryo the seven typical rhombomeres are completely developed. Each rhombomere is related to the developing cranial

nerves. The first or cerebellar rhombomere is shallow. The second is characterized by a broad region and is related to the trigeminal nerve, whereas the facial nerve attaches to the fourth rhombomere. The otic vesicle, and 9th and 10th cranial nerves are related to the 5th, 6th, and 7th rhombomeres respectively. The cerebral vesicles are distinct at 25 to 30 somites and the corpus striatum is a distinct mass ventrally in the forebrain at the 40-somite stage at 13 1/2 days (Hicks et al., 1957).

Development of the cerebral cortex of the rat has also been investigated by means of the autoradiographic technique after injection of tritiated thymidine between the 16th day of gestation and the first day of birth (Berry et al., 1964; Berry and Rogers, 1965). The neuroblasts, which derive from division of the primitive ependymal cells, form three successive waves of migration. The first one, at 17th day of gestation reaches the granular layers V and VI, the second, which forms on the 18th day, populates layer IV. Those neuroblastic cell waves, which form on days 19, 20, and 21, migrate through the deeper layer of the cortex and form layer II and III. As development of the embryo progresses, varying processes of neural cell proliferation, migration, and differentiation occur. Cell layers and other parts of the brain differentiate from the germinal epithelial cells.

At about 16-16 1/2 days or 28 somites standard stage of Altman and Dittmer (1962), the metencephalic dorsal plate (cerebellum) thickness. The cerebellum consists of five transverse deep folds and two shallow ones when the fetus reaches its term. It consists primarily of four layers, namely: the outermost outer granular layer, molecular layer, Purkinje cell layer, and the innermost inner granular layer (Addison, 1911). Both



Addison (1911) and Das and Nornes (1972) sequentially studied neurogenesis in the cerebellum of prenatal and postnatal rat. The earliest nerve cells to form are the Purkinje cells on day 15 to 16 of gestation. The outer granular layer at term is composed of several rows of round cells or dark staining oval nuclei. The molecular layer is narrow and serves as the site of the migration of cells from the outer granular layer. The Purkinje cells are arranged in a layer of 2-3 irregular rows with oval nuclei. The inner granular layer is derived from two sources, the outer granular layer and the mantle layer.

Many factors have been found involving the CNS abnormalities. These factors are nutritional factors, hormones, drugs, physical factors, and infectious agents.

#### Nutritional Factors

##### Hypovitaminosis and hypervitaminosis A

Hypovitaminosis and hypervitaminosis A are found teratogenic to the developing central nervous system. Hydrocephalus occurring in young rabbits was found as a direct consequence of vitamin A deficiency (Millen et al., 1953). The gross malformation showed dilation of the lateral and third ventricles. The cortex and white matter were reduced to a thin layer lining the interior of the skull in some cases. Evidence suggested that stenosis of the cerebral aqueduct is the primary cause of hydrocephalus resulting from vitamin A deficiency. Harrington and Newberne (1968) found that maternal blood levels induced by vitamin A deficiency in rabbits are correlated with hydrocephalus of newborn cases. The indication of hydrocephalus first occurs when serum concentration of vitamin A

is about 35  $\mu\text{g}/100\text{ ml}$ , and reaches 100 percent of incidences at 20  $\mu\text{g}/100\text{ ml}$ . In the pig, if the deficiency of vitamin A is induced throughout the second and third trimester of pregnancy, newborns are stillborn and reveal various degrees of internal hydrocephalus and myelocoele observed at post mortem examination (Palludan, 1970).

Excess of vitamin A is also detrimental to the embryonic development of the rat. Excessive intake of vitamin A (35,000 units in daily diet from 2nd, 3rd, or 4th to 16th day post conception) results in exencephaly, an extrusion of the brain covered with a thin membrane to the external surface of the head. This cranial deformity occurs consistently (Cohlan, 1953). In some litters, hydrocephalus is characterized by enlarged dome-shaped head with increased transillumination. Cohlan (1954) found that the period from the 7th to 10th day of gestation is most susceptible to the teratogenic effect of hypervitaminosis A. Spina bifida with meningocele was also reported in his study. Geelen (1973) indicated that fetal rat exencephalosis induced by hypervitaminosis A has a skullbase malformation. He concluded that the nonclosure of the neural plate causes abnormal flexure in the developing brain and results in abnormality of the skullbase. Unlike Geelen (1973), Theodosius and Fraser (1973) postulated that closing the neural tube in the mouse depends upon a particular configuration of neurectodermal cells forming the brain and spinal cord and the presence of sufficient supportive mesenchymal cells.

#### Riboflavine deficiency

Riboflavin is a component of the coenzyme flavin mononucleotide and flavin adenine dinucleotide, which are necessary growth factors for ani-

mals. The riboflavin deficient, galactoflavin feeding 60-90  $\mu$ g beginning on day 9-1/3 or 10-1/3 of gestation for 96 hours induced various malformations in mice. Skeletal and soft tissue abnormalities including hydrocephalus were first observed on day 13 after mating (Kalter, 1963). Ventricular dilation and the surrounding nervous tissues of the 4th ventricle were the main areas affected. The riboflavin deficiency was assumed to interfere with certain enzymic reactions required for skeletal differentiation. Studying in the rat, Shepard et al. (1968) explained that the possibility of congenital defects may result from death of mesenchymal cells and neural tissue, which have a particular riboflavin requirement. Cellular deaths are quite common if there is a marked decrease in the ability of the electron transport system where cellular demand for energy increases due to a high rate of cellular multiplication (Aksu et al., 1968).

#### Folic acid deficiency

Folic acid or pteroylglutamic acid is a known antianaemic factor. Fetal development in the rat is severely affected if pteroylglutamic acid deficiency is induced for 36 hours after day 8 to 9-1/2 of gestation. This period is most conducive to cardiovascular defects, hydrocephalus, diaphragmatic defect, and gastroschisis (Nelson et al., 1956). Thiersch (1954) used certain antagonist of folic acid, 2, 4--diaminopyrimidine to study the teratogenic effect on the pregnant rat. Malformations were mostly on the developing brain. The brain without meninges was exposed through the cranial defect if the folic acid antagonist was administered prior to implantation. On the other hand, abnormalities consisted of internal hydrocephalus, under-developing cranial bones, and microcephalus;

failure of the cranium to close over the brain occurred when the drug was administered during implantation. Stempak (1965) used another folic acid antagonist, x-methyl folic acid and 9-methyl pteroylglutamic acid for 48 hours period from day 8 of pregnancy. Hydrocephalus was the only defect reported. The addition of vitamin B<sub>12</sub> to the folic acid deficient diet either by injection or in the diet could prevent the hydrocephalus (O'Dell et al., 1951).

The cause of hydrocephalus in the fetus induced by folic acid deficiency was occlusion of the cerebral aqueduct (Overholser et al., 1954; Stempak, 1965). Occlusion of the cerebral aqueduct was apparent from absence of a specialized group of columnar ependymal cells in the roof of the cerebral aqueduct, caudal to the 3rd ventricle.

#### Niacin deficiency

Deficiency of niacin or nicotinic acid has been known in causing pellagra in man or black tongue in dog. Multiple congenital defects resulting from acute maternal niacin deficiency during pregnancy in the rat were investigated by Chamberlain and Nelson (1963a). Niacin antimetabolite, 6-aminonicotinamide (6-AN) was administered at 100 mg/kg of body weight on day 7-9, 8-10, 9-11, or 10-13 of gestation. Days 8-10 was the period of highest sensitivity, which produced 80% of embryonic mortality and abnormalities. They included defects of the central nervous system, skeleton, and the urinary tract. Hydrocephalus was also observed following injection of 6-AN from days 10 through 20 of pregnancy (Chamberlain and Nelson, 1963b). The evidence of hydrocephalus was characterized by the thinning of the ventricle and the cerebral cortex. The massa intermedia and corpus callosum were sometimes absent or reduced in size. His-

tological and histochemical analyses indicated hemorrhage of the central nervous system, and transitory increase of glycogen in the hypoplastic choroid plexus (Chamberlain, 1970). In the hamster multiple defects including exencephaly and hydrocephalus occurred if 5  $\mu$ g of 6-AN were given intra-amniotically on days 8 and 9, and 50-100  $\mu$ g on day 9 of gestation (Turbow et al., 1971). Chamberlain (1972) applied the scanning electron microscopic technique to study the ventricular surface of the fetal rat treated with 6-AN between day 13-21 of gestation. There were signs of undifferentiation and growth of microvilli and cilia on the surface of ependyma and choroid plexuses. Some of the flattened cells had ruptured plasma membranes.

#### Pantothenic acid deficiency

Pantothenic acid was found in a combined form with coenzyme A in the tissue. The congenital malformations of pantothenic acid induced by its antimetabolic, omega-methylpantothenic acid during the second week of pregnancy were similar to the deficiency of either pteroylglutamic acid or riboflavin of the same dosing period (Nelson et al., 1957).

#### Vitamin E deficiency

Vitamin E was first recognized as a fertility factor in rats. Cheng et al. (1957) observed gross malformations of the developing embryos induced by maternal vitamin E deficiency with 2 mg of d, 1-alpha-tocopherol acetate on the 10th day of gestation. By the 13th day exencephalus was recorded. The abnormal bone development involved the parietal bones, which were absent or underdeveloped in either case of hydrocephalus or exencephaly (Cheng and Thomas, 1953). Exencephaly was probably caused by the non-closure of the midbrain (Cheng et al., 1957).

### Purine and pyrimidine antimetabolites

Antimetabolites of DNA have been studied for their teratogenic effect on the central nervous system. In rats, neurons in the fetal forebrain and spinal cord were seen after administration of 6-mercaptopurine (Adhami and Noack, 1975). All fetuses showed a hydrocephalus externus at the dependent dose level 50 mg/kg of body weight on day 12 of pregnancy. This suggested that this purine antagonist, which inhibited the synthesis of DNA and RNA may injure the connective tissue and cartilaginous and bony structures of the skull leading to the obstruction of the venous and cerebrospinal fluid drainage from the brain.

Many pyrimidine antimetabolites affect the developing brains of both rats and mice. Skalko et al. (1971) found exencephaly associated with micrognathia and open eyelids as the only defects in mice produced by treatments with 5-bromodeoxyuridine (300-500 mg/kg), a thymidine analogue, on day 7 of pregnancy when the rostral neuropore is still open. If 5-bromodeoxyuridine is administered on 13th - 15th day of pregnancy, the mitotic figures showed abnormal chromosomal structure and disappeared after migration to the cerebral cortex (Langman et al., 1972). In the fluorodeoxyuridine-treated fetuses, the neuroepithelial cells and the Purkinje cells of the cerebellum lost their interkinetic movements and cell division (Langman et al., 1972, 1973). Though these Purkinje cells migrated towards their definite place, they disappeared later and resulted in a small number as compared to the controls. Pyrimidine analogue, 5-iododeoxyuridine, was reported as a teratogenic agent in both rats (Percy and Albert, 1974) and mice (Percy, 1975). It caused persistence of granular cells in the external granular layer of the cerebellum of three con-

secutive dosing periods beginning on day 16 to 18 of pregnancy while Rodier et al. (1973) found degenerating cells in external granular layer when animals were treated with 5-Azacytidine. The developing brain of the fetal rat was highly sensitive to 5-fluorouracil (Kameyama et al., 1972). Both undifferentiated proliferating cells and migrating cells exhibited degenerative changes. About six percent of the fetuses showed encephalocele and gastroschisis if 6-Azauridine was given at the lowest dose of 12 mg/kg of body weight on days 6-12 of pregnancy.

### Metals

In the past, mercury was used effectively as a fungicide for seed preservation. Minimata disease in Japan had been reported causing nervous defects in the newborn babies due to their mothers consuming fish contaminated with high concentrations of methylmercury discharged from the industrial waste. Methylmercury, one of the most toxic compounds of mercury, causes neurological and behavioral disturbance (Weiss and Dorothy, 1975). The occurrence of severe central nervous system disturbance in newborn babies by methylmercury indicates the mercury level in the brain of 2-4 ppm dry weight (King et al., 1976). Organic mercury can cross the placenta and blood brain barrier to disturb the normal development of the fetal brain. Spyker and Smithberg (1972) injected methylmercury dicyandiamide (MMD) intraperitoneally in mice on days 6-13 of pregnancy, which was embryolethal, causing retarded growth and abnormality of the brain, resulting in exencephaly and encephalocele. Postnatal mice treated with methylmercury during the 6th to 17th day of gestation showed inhibition of many enzymes in the area corresponding to the fetal site of the

tion of many enzymes in the area corresponding to the fetal site of the internal granular layer of the cerebellum (Khera and Nera, 1971).

The toxicity of methylmercury was also demonstrated in the cat (Khera et al., 1974). The cerebrum was characterized by edema, spongy appearance of the gray matter, and neuronal pyknosis; also, the granular cells in the cerebellum were decreased and appeared pyknotic.

Cadmium chloride at the dose level of 16  $\mu\text{m}/\text{kg}$  of body weight was found highly teratogenic when given intraperitoneally to pregnant rats (Barr, 1973). Hydrocephalus frequently occurred with few cases of exencephaly where cadmium chloride was given on days 9 and 10 of gestation. Semba et al. (1973), on the other hand, found that intraperitoneal injections of cadmium sulphate at the dose level of 5 mg/kg to pregnant mice on day 7 of gestation caused exencephaly in about 50 percent of the litter. The earliest sign of nonclosure of the neural tube, resulting in exencephaly, is about the 17-somite stage or day 9 of gestation. The low dose of cadmium sulphate, 1 mg/kg, can prevent exencephaly (caused by high dose of cadmium sulphate) if treatment is given one day prior to administration of a higher dose (Semba et al., 1974).

Congenital communicating hydrocephalus can be induced in the rat on days 9 and 10 of gestation with radioactive tellurium (Agnew, 1972; Agnew et al., 1973). With autoradiographic technique  $\text{Te}^{127\text{m}}$  was demonstrated to cross the placenta and blood brain barrier like cadmium, and accumulated in the choroid plexus. Electron microscope evidence showed tellurium deposited also in the brain cells, choroid plexus epithelium, and other tissues. Tellurium is assumed to induce hydrocephalus by interfering with the secretion or reabsorptive function of the choroid plexus.



Hurley and Sevenerton (1966) and Warkany and Petering (1972) studied the congenital malformations resulting from zinc deficiency. Hydrocephalus was the main sign of brain defect and few cases of exencephaly were also evident. Spinal cord abnormalities were associated with short and abnormal tails (Warkany and Petering, 1972).

### Physical Factors

Some of the physical factors employed in experimental teratology are radiation, hypoxia and temperature. All of these have been proved experimentally to affect the central nervous system.

#### Radiation

Ionizing radiation effect, especially, was one of the agents hazardous to the nervous system during both prenatal and postnatal life. After World War II many Japanese children, whose mothers during pregnancy had been exposed to the atomic bomb in Hiroshima and Nagasaki, developed abnormal central nervous systems. It is reasonable to believe that the effect of irradiation has a direct action on the embryo and fetus, e.g., intrauterine death, malformation, and growth retardation. The central nervous system is particularly susceptible to radiation (Hicks, 1952).

X-ray irradiation (and its effect during the early period of development) was the first environmental factor to be studied in the embryo. Job et al. (1935) found that certain malformations in the rat occurred on a certain day of the gestation period. Hydrocephalus was induced by X-ray irradiation on the 9th day, whereas the eye defects were produced on the 10th day of gestation. Hicks (1954) set the timetable of organogenesis in the rat exposed to the low dose, 100-200 r, of X-ray irradiation. No

malformation was found following irradiation on the first eight days of gestation. A marked change of brain malformation occurred after exposure to X-rays: encephalocele and anencephaly on the 9th day, hydrocephalus and other brain deformities from the 10th to 13th day, and microcephalus or cerebellar deformities on the 12th day until the neonatal period. Wilson et al. (1953) found many types of brain and spinal cord defects on the 10th day after using X-rays. Hydrocephalus, exencephaly, and hypoplasia of the brain were major defects. In hydrocephalus he found fusing of the brain wall and the overlying ectoderm associated with decrease or absence of the vascular tissue although obstruction of the cerebrospinal fluid pathway could not be found. Ectoderm in the developing brain tissue ruptured and formed the exencephaly. Some offspring showed grossly a small elevated area on the head. Dissection revealed a protrusion of meninges and brain tissue through a skull defect, meningoencephalocele. Localized hypoplasia of some parts of the brain, especially in the fore-brain, was identified. Deficiency in growth resulted in distortion of shape and size of the brain as development progressed. Following irradiation on the 10th day, spinal cord defects caused by normal growth from the lateral part of the cord were observed. Shoji et al. (1974) found three types of brain malformations in rat and hamsters. Fetuses exposed to X-rays on the 11th day of gestation showed hypoplasia of the olfactory bulbs. The following days of exposure resulted in development of hydrocephalus and microcephaly, which were similar to the findings of Hicks (1954).

There was a distinct correlation of both degree and site between the abnormal vascular pattern and the histological aberration of the brain cortex (Hayashi et al., 1972). Vulnerability of vessel walls, shown by leakage of the injected ink from vessels, was found as a causal relationship between abnormal vascular formation in the brain and development of hydrocephalus. The degree of malformations in the developing central nervous system depends upon the dose level of ionizing radiation used. Hicks (1950) found no external malformation in the full-term rats when the dosage applied was below 200 r on the 3rd week of gestation. Histologically brains showed severe maldevelopment of the corpus callosum, hippocampus, corpus striatum, and cerebral cortex. On the other hand, the dosage applied between 220-600 r resulted in acute necrosis of the rapidly growing parts of the brain and spinal cord as well as the retina of the eyes. The effect of low levels of ionizing radiation, 10 to 40 r, was also studied in the developing cerebral cortex of the rat on the 16th or 18th day of gestation by D'Agostino and Hicks (1965). There was a slight decrease in the thickness of cortical layer II, III, and VI. Bizarre rosettes and clusters of nerve cells with a few mitotic figures in the center were found in the cerebral cortex (Hicks, 1952). The rosettes were considered to be the result of destruction of the continuity of the neuroectodermal cell layers after irradiation and a sign of regenerative effort by the surviving cells (Hicks et al., 1957; Hicks, 1958). Skalko (1965) concluded that the overall effect of ionizing radiation was caused by suppression of DNA synthesis--the higher the dose the larger the adverse effects.

### Stress

Stress is one of the factors studied to induce malformation of the central nervous system. Offspring of crowded pregnant mice placed in 18 per cages on day 3-4 of pregnancy or parabiotically stressed mice on the 6th, 7th, or 8th day of pregnancy produced open neural folds, exencephaly, and malformation of head and tail (Hamburgh et al., 1972; Hamburgh et al., 1974). The mean of maternal adrenal weights increased significantly to support the experimental results. Offspring from the heat-stressed female guinea pigs on days 20-21 or 22-23 were found markedly microcephalic. If the maternal body temperature was elevated more than 2°-5°C above normal, brain weight was reduced to about 8.4 percent for each 1°C elevation of the body temperature when compared with the controls (Edwards, 1969a). One stillborn with an enlarged cranium accompanied by a severe bilateral hydrocephalus was reported from a female exposed to hyperthermia on days 11-14 of gestation (Edwards, 1969b). The effect of hyperthermia was significantly seen in ewes when exposed to 44°C for nine hours daily during the last two-thirds or the last one-third period of gestation. Cavitation of white matter and micrencephaly were the main abnormalities (Hartley et al., 1974).

### Hypoxia

Hypoxia, by clamping the uterine vessels for 72 hours on 15th to 18th day of pregnancy, reduced myelinization in different parts of the brain and the number of fat-granule-containing cells observed in the fiber tracts (Nakamura, 1975).

### Genetic Factor

Recently the problem of congenital malformations especially in humans has increased greatly. An epidemiologic approach seems to be important for understanding the etiology of congenital malformations. Most congenital malformations are believed to have multiple origins. Many studies have shown that the expression of major genes will be altered by treatment with teratogenic agents (Hamburgh et al., 1970). Rats from Sherman stock behaved as if autosomal recessive mutation had occurred (Layton and Smith, 1968). The affected animals had complete cranioschisis. Exencephaly was found only in mouse fetuses of MT strain; this may be caused by an autosomal recessive gene (Shoji, 1973). The brains of spontaneous hydrocephalic MT mice, 20-40 days old were investigated by electron microscopic technique (Takeuchi et al., 1974). There were some degenerative changes in the myelinated fibers, neurons, ependymal, and glial cells. Burda and Michael (1975) studied the ventricular cells of mouse brains of the homozygous loop-tail gene. The quantitative reduction in the microvilli of the ventricular cells was indicative of the fact that they may have a functional defect of secretory activities.

The vascular bed change was one of the reasons causing hydrocephalus in a recessive mutant gene (Hirayama et al., 1975). Because of venous dilation the vascular bed in the brain increased in the last few days before birth. Rupture of a vein along the ventricular surface may cause ventricular dilation. Trisomy syndrome was induced experimentally by Gropp (1974). Trisomy No. 12 showed exencephaly, mostly without developmental retardation.

## Hormonal Imbalances

The synthetic progestin norethynodrel in combination with the estrogen mestranol is widely used as a contraceptive. Progestin and its metabolites were tested to determine their potential embryotoxicity. Cranial abnormalities, including exencephaly, internal hydrocephalus, and partial cryptorchidism, were produced in fetal mice (Gidley et al., 1970). Internal hydrocephalus involved the lateral and third ventricles if the hydroxy metabolites of norethynodrel were administered on days 8, 9, and 10 of gestation. Incomplete development of the parietal bones was about 64 percent in the fetuses examined. A small percentage showed exencephaly from the drug dependent effect. Either one or three consecutive doses (0.2-2.4 mg/kg) between days 6 and 10 of gestation were also found to be teratogenic in mice of two strains. Hydrocephalus and club feet were the major findings among other abnormalities (Andrew et al., 1972). Exencephaly was not found in rats when treated with similar antifertility hormone as compared to mice (Andrew et al., 1973).

## Drug

In human cases many drugs are prescribed to pregnant women, especially in early pregnancy. Unlike thalidomide, congenital defects caused by drugs are still not clearly known. Aspirin generated a long controversy as to whether it was a teratogenic agent. There is no clear-cut evidence whether pregnant women consumed aspirin only during the early period of pregnancy and delivered abnormal children. In rat, salicylic acid is the only drug found in embryos and maternal serum (Kimmel et al.,

1971). There is a correlation between the central nervous system abnormalities and time of treatment with salicylic acid. For example, at day 9 of gestation when the neural plate is being formed, the defect involved cranioschisis, skeleton, and gastroschisis. At day 10 of gestation, the abnormalities were mostly manifested as hydrocephalus.

An antiepileptic drug, Trimethdione, was reported as teratogenic to humans (German et al., 1970). Early communicating hydrocephalus, cerebral necrosis, and subdural hemorrhage were associated with other abnormalities. In mice, multiple malformations including internal hydrocephalus and exencephaly were produced after a single intraperitoneal dose of 150 mg/kg in early gestation period between day 9 and 10 (Harbison and Becker, 1969). The incidence of diencephalic malformation was found in rabbit and hamster (Gottschewski, 1974) when actinomycin D during the preimplantation stage of pregnancy was administered intravenously. The malformation if caused by impairment of DNA synthesis is questionable.

### Infectious Agents

In humans many infants have multiple malformations of unknown causes, though a few viruses have been shown to cause congenital defects. Presently, rubella and cytomegalovirus have been proven to be teratogenic (Lapinleimu et al., 1972). Congenital rubella, considered as a chronic disease in humans, begins in fetal life and continues into infancy. The epidemic of congenital rubella syndrome occurred in Sweden in 1951. The central nervous system defects involved micrencephaly, anencephaly, hydrocephaly, and menigocele (Lunstrom and Ahnsjo, 1962). Between 1964

and 1965 another outbreak was reported in the United States. About 3 out of 1000 children born had evidence of congenital rubella syndrome (Singer et al., 1967). Leptomeningitis and many small foci of necrotic areas, especially in the white matter, basal ganglia and the mid-brain, were found in most cases. In the area of necrosis, Rorke and Spiro (1967) found characteristic vascular damage of one or more layers of the vessel wall with replacement of normal tissue. Desmond et al. (1967) also found encephalitis, multiple necrotic areas, and vascularitis in infected children between birth and 18 months of age in 81 out of 100 patients. They concluded that rubella was an etiological agent in the malformation of neurological disorders in prenatal life. In rat intracardial injections with rubella virus on the 5th or 6th day of gestation induced congenital malformations of the brain characterized by hydrocephalus, marked narrowing of the cerebral aqueduct and parts of the third ventricle, and adhesion of the meninges to the base of the brain (Cohlan and Stone, 1955). The mechanism of teratogenesis for rubella was due to direct infection of the virus in certain tissue of the developing fetus while the indirect effect may be caused by vascular occlusion resulting from the damage of blood vessels (Sever and London, 1969).



## MATERIALS AND METHODS

Twenty-five germ free, virgin Wistar (Cesco Lab. Company, Omaha, Nebraska) female rats were paired with males. Vaginal smears were taken the following morning and females with positive sperm in smear were considered pregnant. A sperm-positive smear on the following day was assigned as day 1 of pregnancy. The rats were caged individually and provided with food "Teklad," which was mouse and rat diet, containing 4 percent of fat manufactured by Teklad Mills, Iowa. Water was provided ad libitum. The calculated nutrient composition is presented in Table 1. Five rats were assigned randomly as controls and the rest were divided into experimental groups of 20. A single oral dose of 1.6 percent aqueous solution of ETU was given by intubation on day 12 of gestation at the rate of 300 mg/kg of body weight to the experimental group; the control group was dosed with distilled water only.

Pregnant female rats were sacrificed by carbon dioxide gas on day 22 of gestation. Weight and number of fetuses, resorption sites, and the maternal weights before and after sacrifice were recorded. Corpora lutea were counted under the dissecting microscope. The average weight of fetuses was determined. Fetuses were fixed in 10 percent buffered neutral formalin and Bouin's fixative. For better fixation the abdomen of all fetuses was opened with scissors just caudal to the umbilicus. Grouped fetuses of similar brain lesions were studied grossly in both the mid-sagittal and dorsal plane with thin razor blade. In exencephaly, the prominent protrusion was measured in 3 dimensions (width x length x height). The thickness of dorsal plane was approximately 1 cm. Cranial

Table 1. Calculated nutrient composition<sup>a</sup>

		Teklad mouse/rat diet (4% fat)
Protein	%	25.92
Fat	%	4.15
Fiber	%	4.56
Ash	%	9.88
Moisture	%	10.23
Nitrogen free extract	%	43.74
Gross energy	Kcal/Kg	3844.00
Minerals		
Calcium	%	2.06
Phosphorus	%	0.92
Sodium	%	0.63
Chlorine	%	0.63
Potassium	%	0.96
Magnesium	%	0.19
Cobalt	mg/Kg	0.65
Copper	mg/Kg	17.31
Iodine	mg/Kg	1.76
Iron	mg/Kg	224.00
Manganese	mg/Kg	85.27
Zinc	mg/Kg	27.87
Vitamins		
Vitamin A	IU/Kg	3996.00
Carotene	mg/Kg	3.52
Vitamin D <sub>3</sub>	IU/Kg	1540.00
Vitamin E	IU/Kg	12.27

<sup>a</sup>Teklad Mills, Winfield, Iowa 52659.

Table 1. (Continued)

		Teklad mouse/rat diet (4% fat)
Vitamin B <sub>12</sub>	mg/Kg	0.0109
Vitamin C	mg/Kg	---
Choline	mg/Kg	1710.00
Folic acid	mg/Kg	0.4252
Menadione	mg/Kg	12.14
Niacin	mg/Kg	38.51
Pantothenic acid	mg/Kg	12.36
Pyridoxine	mg/Kg	5.47
Riboflavin	mg/Kg	7.44
Thiamine	mg/Kg	4.36
Amino acids (% of diet)		
Arginine	%	1.72
Cystine	%	0.35
Histidine	%	0.60
Isoleucine	%	1.25
Leucine	%	2.01
Lysine	%	1.54
Methionine	%	0.37
Phenylalanine	%	1.20
Threonine	%	0.96
Tryptophan	%	0.30
Tyrosine	%	0.64
Valine	%	1.31

sections of midsagittal and dorsal planes and spinal cord sections of three levels (e.g., cervical, thoracic, and lumbar regions) were processed according to the standard histological procedures. Chloroform was used as the dehydrating agent. Embedded tissues were cut in serial sections of 10  $\mu$ m thick in both planes. Every sixth section of the paraffin embedded serial sections was mounted; the rest were kept for special staining. Sections were stained routinely with hematoxylin and eosin.<sup>1</sup> Two special staining techniques applied were aldehyde-thionin-PAS<sup>2</sup> and Masson trichrome technique (Ralis et al., 1973). The staining solution and the procedures are as follows:

Hematoxylin and eosin

1. Stock eosin

Eosin Y	1 gm
Distilled water	100 ml

2. Stock phloxine

Phloxine B	1 gm
Distilled water	100 ml

3. Working solution

Stock eosin	50 ml
Stock phloxine	5 ml
95% alcohol	390 ml
Glacial acetic acid	0.5 ml

---

<sup>1</sup>From the Department of Veterinary Anatomy, Pharmacology, and Physiology, Iowa State University.

<sup>2</sup>From Armed Forces Institute, Washington, D.C.

## 4. Harris hematoxylin solution

Hematoxylin	5 gm
Absolute ethyl alcohol	50 ml
Potassium alum	100 gm
Mercuric oxide	2.8 gm
Distilled water	1000 ml

The solution was added with 2-4 ml of glacial acetic acid per 100 ml before use.

## 5. Saturated lithium carbonate

Lithium carbonate	1 gm
Distilled water	100 ml

Staining procedure

1. Deparaffinized sections with two changes of xylene, each 5 min.
2. Hydrate sections by taking through absolute alcohol, 95% alcohol, 70% alcohol to distilled water, each 2 min.
3. Stain in hematoxylin for 8 min.
4. Wash in running tap water until the section is blue.
5. Differentiate in 1% acid alcohol 1-2 dips.
6. Leave in running water for 3-5 min.
7. Blue the nuclei with saturated lithium carbonate 1 min.
8. Wash in running water 5 min.
9. Stain in eosin working solution 2 min.
10. Differentiate the eosin in 70% alcohol 2 min.
11. Dehydrate sections by taking through 95% alcohol and two changes of absolute alcohol, 2 min. each.

12. Clear in xylene 2 changes, 2 min. each.

13. Mount with Permount.

Result

Nuclei	blue
Red blood cells	red
Most other tissue components	pink

Aldehyde-thionin-PAS

1. 0.5% sulfuric acid solution

Conc. sulfuric acid	0.8 ml
---------------------	--------

Distilled water	99.5 ml
-----------------	---------

2. 0.5% potassium permanganate solution

Potassium permanganate	0.5 gm
------------------------	--------

Distilled water	100 ml
-----------------	--------

3. 2% potassium metabisulfite solution

Potassium metabisulfite	2.0 gm
-------------------------	--------

Distilled water	100 ml
-----------------	--------

4. Aldehyde thionin solution

Thionin	0.5 gm
---------	--------

70% alcohol	99.5 ml
-------------	---------

Paraldehyde	7.5 ml
-------------	--------

Conc. hydrochloric acid	1.0 ml
-------------------------	--------

5. 0.5% periodic acid solution

Periodic acid	0.5 gm
---------------	--------

Distilled water	100 ml
-----------------	--------

## 6. 1% orange G solution

Orange G	1 gm
----------	------

Distilled water	100 ml
-----------------	--------

## 7. 1% glacial acetic solution

Glacial acetic acid	1 ml
---------------------	------

Distilled water	99 ml
-----------------	-------

## 8. 5% phosphotungstic acid solution

Phosphotungstic acid	8 gm
----------------------	------

Distilled water	100 ml
-----------------	--------

## 9. Schiff's reagent solution

Bring 200 ml of distilled water to boil. Remove from flame and add 1 gm of basic fuchsin and stir until dissolved. Cool to 50°C, then filter. Add 20 ml of 1 N hydrochloric acid. Cool to 25°C, then add 1 gm of sodium metabisulfite. Place in the dark in a stoppered bottle for 24 hours. Solution has a hay color at this time. Add 0.5 gm of activated charcoal and shake well, then filter and store in a brown bottle.

Staining procedure

1. Deparaffinize sections with two changes of xylene, 5 min each.
2. Hydrate sections by taking them through absolute alcohol, 95% alcohol, 70% alcohol to distilled water, 2 min each.
3. Oxidize the sections in equal part of 0.5% sulfuric acid and 0.5% potassium permanganate for 2 min.
4. Bleach with potassium metabisulfite 1 min.
5. Rinse well in distilled water.
6. Stain in aldehyde thionin solution in an air stoppered container for 50 min.

7. Rinse in distilled water.
8. Oxidize in 0.5% periodic acid solution 5 min.
9. Rinse in distilled water.
10. Stain in Schiff's reagent 15 min.
11. Wash in running water 15 min.
12. Stain in orange G solution 3 min.
13. Dip in phosphotungstic acid solution 1 min.
14. Rinse briefly in glacial acetic acid solution.
15. Dehydrate sections by taking through 95% alcohol and 2 changes of absolute alcohol, 2 min each.
16. Clear in xylene 2 changes, 2 min each.
17. Mount with Permount.

#### Result

Nissl substances	blue-black
PAS positive material	red
Background	depends on differentiation
Nuclei and red blood cells	orange

#### Goldner's modification of the Masson trichrome technique

##### 1. Weigert's iron hematoxylin

###### Solution A

Hematoxylin	1 gm
Absolute alcohol	100 ml

###### Solution B

30% aqueous ferric chloride	4 ml
Distilled water	100 ml



Hydrochloric acid            1 ml

Mix equal volumes of solution A and B before use.

2. Solution A

1% Ponceau 2R in 1%  
acetic acid                      2 parts

1% acid fuchsin in  
1% acetic acid                      1 part

3. Solution B

Phosphomolybdic acid            4 gm

Orange G                            2 gm

Distilled water                    100 ml

4. Solution C

Light green                        0.1 gm

0.2% acetic acid                    100 ml

Staining procedure

1. Deparaffinize sections with two changes of xylene, 5 min. each.
2. Hydrate sections by taking through absolute alcohol, 95% alcohol, 70% alcohol to distilled water, 2 min. each.
3. Stain nuclei with Weigert's hematoxylin 20 min.
4. Wash in running water, blue.
5. Differentiate in 1% acid alcohol 1-2 dips.
6. Wash in running water, blue for 3-5 min.
7. Stain in solution A, 5 min.
8. Differentiate and mordant in solution B, 10 min.
9. Rinse in 1% acetic acid.
10. Stain in solution C for 5 min.
11. Rinse in 1% acetic acid.

12. Dehydrate sections by taking through 95% alcohol and 2 changes of absolute alcohol, 2 min. each.
13. Clear in xylene 2 changes, 2 min. each.
14. Mount with Permount.

#### Result

Nuclei	blue-black
Red blood cells	red
Muscle, fibrin	pink
Collagen, mucin	green
Myelin	purplish brown

The control and hydranencephalic brains were studied by a scanning electron microscope by using 10  $\mu$ m thick paraffin sections. Sections were mounted on 22 x 22 glass cover slips and processed for hematoxylin and eosin stain. Then they were mounted on the 2 x 2 cm brass disc and attached on the stub with conducting material, tube paint. Carbon and gold were evaporated in a vacuum evaporator at  $10^{-5}$  to  $10^{-6}$  torr, used to prevent charging effect by the electron beam. The sections were viewed at  $45^\circ$  towards the beam (McDonald et al., 1967). After the sections had been viewed and photographed with the scanning electron microscope, the cover slips were detached from the brass with acetone, dehydrated with alcohol, and cleared in xylene. The cover slips with sections on them were mounted with Permount for light microscopic study.

## OBSERVATION

The results from the experiment have revealed that a single oral administration of ETU at the dose level of 300 mg/kg, given to female rats on day 12 of pregnancy, resulted in abnormalities averaging 88.27% of the total experimental rats. Gross malformations involved the head, face, limbs, and tail. None of these anomalies were observed in the control group. The abnormalities were varied not only in the same litter but also among the litters. ETU did not affect the fertility rate nor the resorption rate in the experimental group as compared to the controls (Table 2). ETU caused growth retardation of those fetuses that showed at least one abnormality. The average weight of the affected ones was  $3.86 \pm 0.57$  grams as compared to the mean weight of the controls,  $5.40 \pm 0.36$  grams (Table 3).

The cranial defects were identified as 38% exencephaly, 30% hydrocephalus and 20% hydranencephaly. In case of exencephaly (Figures 20-22 and 37-39) the amount of brain protrusions varied through the defect of frontal, parietal, and occipital bones. The defects in bones were proportional to the protrusions of the brain. Most of the brain surface was irregular and sometimes associated with hemorrhage. The size of brain protrusion was  $0.2 \times 0.3 \times 0.05$  cm up to  $1.0 \times 1.1 \times 0.6$  cm. The gross frontal sections in Figures 22 and 39 and midsagittal sections in Figures 21 and 38 did not reveal any fluid-filled cavity in the defective brain.

Hydrocephalus was found in fetuses that had dome-shaped heads, with or without cysts (meningoencephalocele) in the occipital region (Figures 75-77) and these had normal appearance of the head with short or absent tail (Figures 57-59). Gross midsagittal sections (Figure 98) and dorsal

Table 2. Teratogenic effects of ETU at the dose level 300 mg/kg given orally to the rats on day 12 of gestation<sup>a</sup>

Exp. rat No.	Weight gm D <sub>12</sub>	Weight gm BC <sup>b</sup>	Weight gm AC <sup>c</sup>	Pups/ litter	Resorp- tion site	No. CL <sup>d</sup>
1	275	318	275	9	1	10
2	200	274	220	10	-	10
3	237	312	233	12	-	12
4	288	360	305	10	-	11
5	222	274	234	7	3	11
6	213	300	230	10	-	10
7	238	328	268	12	-	12
8	240	330	260	11	-	12
9	208	279	226	11	-	12
10	263	332	293	10	-	13
11	303	390	350	8	-	18
12	255	320	278	9	2	12
13	245	348	275	8	-	11
14	276	358	295	11	-	11
15	220	255	219	9	2	11
16	290	353	315	7	-	12
17	232	315	263	10	-	11
18	192	254	210	9	1	11
19	253	347	268	13	-	13
20	245	348	287	10	-	12
Control						
21	218	265	230	6	2	8

<sup>a</sup>Total fetuses = 240, experimental fetuses = 196, control fetuses = 44.

<sup>b</sup>BC = before Caesarean.

<sup>c</sup>AC = after Caesarean.

<sup>d</sup>CL = corpus luteum.

Table 2. (Continued)

Exp. rat No.	Weight gm D <sub>12</sub>	Weight gm BC	Weight gm AC	Pups/ litter	Resorp- tion site	No. CL
22	205	278	220	8	1	9
23	242	340	250	9	-	9
24	295	372	298	12	-	12
25	220	245	182	9	-	10

sections (Figure 99) of dome-shaped fetuses revealed exencephalus and hydrocephalus at the same time if the cyst (meningoencephalocele) in the occipital region of the brain was greater than 0.3 cm. Microscopic identification to confirm hydrocephalus must be done for those fetuses that have normal appearance of the head with an abnormal tail.

Hydranencephalic fetuses were easily recognized (Figure 117). They were characterized by the round head, with bulging forehead, and a transparent fluid-filled brain cavity. Incomplete osteogenesis mostly involved the individual or both parietal bones and sometimes the occipital bone where the brain protrusion was often seen. If the brain was severely damaged, the occipital bone was always involved and had a v-shaped defective area. The gross midsagittal and dorsal sections (Figures 118 and 119) showed nearly complete absence of the cerebral hemispheres and fluid filled cavity, which sometimes was hemorrhagic. There was a correlation between the larger brain area damaged and the more incomplete osteogenesis of the skull bones.

Table 3. Effect of ETU on the fetal weight at the dose level 300 mg/kg given orally to the rat on day 12 of gestation

Exp. rat No.	Pups/litter	Litter Wt. gm	Avg. Wt./fetus <sup>a</sup> gm
1	9	25	2.78
2	10	38	3.80
3	12	46	3.83
4	10	32	3.2
5	7	25	3.57
6	10	40	4.00
7	12	49	4.08
8	11	45	4.09
9	11	40	3.63
10	10	33	3.80
11	8	38	4.75
12	9	39	4.33
13	8	33	4.75
14	11	50	4.55
15	9	26	2.89
16	7	31	4.43
17	10	32	3.2
18	9	34	3.78
19	13	75	5.77 <sup>b</sup>
20	10	61	6.10 <sup>b</sup>
Control			
21	6	33	5.5
22	8	38	4.75

<sup>a</sup>The average weight of experimental fetuses =  $3.86 \pm 0.57$  gm; the average weight of control fetuses =  $5.40 \pm 0.36$  gm.

<sup>b</sup>Weight did not include an average.

Table 3. (Continued)

Exp. rat No.	Pups/litter	Litter Wt. gm	Avg. Wt./fetus gm
23	9	48	5.33
24	12	68	5.67
25	9	52	5.77

One hundred and ninety-six fetuses from the experimental rats were observed for distribution of anomalies as summarized in Table 4 and description for each litter is as follows:

Litter No. 1

Fetal brains showed different types of cranial abnormalities, such as exencephaly, hydrocephalus, and hydranencephaly. Seven out of nine fetuses had exencephaly. The brain protrusion was in the range of 0.3 x 0.4 x 0.2 cm up to 0.6 x 0.8 x 0.3 cm. Incomplete osteogenesis of parietal bones was greatly affected in each hydrocephalus and hydranencephaly. Agnathia, cleft lip, ectrodactyly, and absence of tail were associated with all types of cranial defects.

Litter No. 2

All fetuses of this litter showed normal appearance of the head, although growth retardation was found. The average body weight of the litter was 3.80 grams. The only abnormality found was a short or absent tail.

Table 4. Incidence of gross malformations of the fetal rats treated with 300 mg/kg given orally on day 12 of pregnancy

Litter No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Total fetuses	9	10	12	10	7	10	12	11	11	10	8	9	8	11	9	7	10	9	-	-
Exencephaly	7	-	10	3	-	8	-	1	-	8	4	7	4	9	9	1	-	3	-	-
Hydrocephalus	1	10	-	1	7	2	12	10	11	2	-	-	3	2	-	-	-	-	-	-
Hydranencephaly	1	-	2	6	-	-	-	-	-	-	4	2	1	-	-	6	10	6	-	-
Micrognathia	-	-	8	-	-	-	-	11	11	-	1	5	8	-	-	-	10	-	-	-
Agnathia	9	-	10	10	7	10	-	-	-	10	7	4	-	10	9	7	-	9	-	-
Facial cleft	-	-	-	10	-	-	-	-	-	10	-	-	-	-	9	-	-	-	-	-
Cleft lip(s)	9	-	10	-	4	10	-	-	-	-	6	2	1	-	-	-	-	-	-	-
Phocomelia	-	-	-	10	-	-	-	-	-	10	-	-	-	-	9	-	-	10	-	-
Ectrosyndactyly	1	-	4	-	1	3	-	-	-	1	-	-	-	3	1	-	-	-	-	-
Ectrodactyly (forelimb)	9	-	8	10	3	4	-	-	4	10	8	3	3	5	9	7	10	9	-	-
Ectrodactyly (hind limb)	-	-	-	10	-	-	-	-	-	6	3	-	-	-	7	7	10	9	-	-
Polydactyly	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-
Short tail	-	8	-	-	3	3	7	-	3	1	-	1	4	1	1	4	3	6	-	-
Absent tail	9	2	10	10	2	7	3	11	8	9	8	8	4	10	7	3	7	3	-	-



Litter No. 3

Salient abnormality in this group consisted of ten exencephaly and two hydranencephaly related to the defects in the skull bone. A small, round and transparent area, 0.5 cm, was in the center where two parietal bones joined together. Brain protrusion, oriented in rostrocaudal direction was 0.1 x 0.5 x 0.5 cm up to 0.8 x 0.7 x 0.5 cm. The surface of the brain was irregular and highly vascularized, except in two fetuses, which had the smooth surface with a cross band between the occipital and parietal bones. Defects in frontal and occipital bones were involved when more brain protrusion occurred. All fetuses showed agnathia, cleft lips, ectrodactyly, and absence of tail.

Litter No. 4

Six out of ten fetuses exhibited hydranencephaly. A small, round area of parietal bones was unossified in the center of the head, the area being greater than 0.3 cm. The occipital bone also had a v-shaped defect. The rest of the fetuses had exencephaly with a small sized protrusion of brain tissue measuring 0.3 x 0.3 x 0.05 to 0.5 x 0.8 x 0.10 cm and one hydrocephalus. Facial cleft, agnathia, phocomelia, and absence of tail were present in all the fetuses.

Little No. 5

All seven hydrocephalic fetuses were characterized by a dome-shaped head. Two of them had cyst formation (meningoencephalocele) in the occipital region. Internal hemorrhage in the brain cavity was found in one fetus. In a few cases, there was a narrow band of bone across and

between the parietal and occipital bones. Fetuses had agnathia associated with cleft lips, as well as short and absent tails.

#### Litter No. 6

Eight out of the ten fetuses showed exencephaly. The size of the brain protrusion was 0.2 x 1 x 0.1 cm to 0.5 x 1.1 x 0.4 cm. Incomplete osteogenesis was found in both parietal and occipital bones as more brain tissue protruded out of them. Two fetuses had hydrocephalus with cyst forming (meningoencephalocoele) in the occipital region, 0.1 and 0.2 cm diameter. Agnathia and cleft lips were a constant anomaly in every fetus. The number of absent-tails was higher than short tails.

#### Litter No. 7

All twelve fetuses in this litter had normal appearance of the heads, except one that showed incomplete osteogenesis in both the parietal bones (0.1 cm diameter). The only abnormality was either short or absent tail. The average body weight of this litter was 4.09 grams.

#### Litter No. 8

Ten fetuses with cranial defects showed hydrocephalus with cyst (meningoencephalocoele) in the occipital region. The diameter of the cyst (meningoencephalocoele) was 0.2 to 0.3 cm. Only one fetus of this litter had exencephaly. The size of brain tissue protruded was 0.6 x 0.8 x 0.3 cm with irregular surface. All fetuses had micrognathia and absence of tail.

#### Litter No. 9

All eleven hydrocephalic fetuses were characterized by dome-shaped heads with cysts (meningoencephalocoele) in the occipital region similar to litter No. 8. Micrognathia occurred in all the fetuses. Few of them

showed ectrodactyly in the forelimb. Absence of tails was higher than short tails.

#### Litter No. 10

Two out of the ten fetuses were affected by hydranencephaly. A round, transparent area, 0.5 cm in diameter, was in the center of the head. The rest of the fetuses showed exencephaly. The surface of the protruded brain in exencephaly was irregular and oriented in rostro-caudal direction. The protrusion size of the brain tissue was in the range of 0.5 x 0.6 x 0.1 and 0.7 x 0.9 x 0.4 cm. Occipital bone defect was associated with those fetuses having more brain tissue proliferation. Every fetus revealed facial cleft, agnathia, phocomelia, and ectrodactyly. One of them had a short tail; the rest of the litter had no tail.

#### Litter No. 11

The incidence of hydranencephaly and exencephaly was equal. Four hydranencephalic heads were similar to those in fetuses in rat No. 10. The size of protruded brain in exencephaly varied from 0.4 x 0.5 x 0.1 to 0.5 x 0.9 x 0.5 cm extending through the defective area of the parietal and occipital bones. All fetuses had agnathia and ectrodactyly in the forelimbs. Six of them showed cleft lips.

#### Litter No. 12

Two out of the nine fetuses exhibited hydranencephaly with cysts (meningoencephalocoele) in the occipital region. The brain tissue of exencephaly protruded through the defective parietal and occipital bones. The size of brain protrusion was 0.5 x 0.6 x 0.6 to 0.6 x 0.8 x 0.5 cm. Each abnormality, micrognathia, and agnathia occurred in equal numbers. Two fetuses had cleft lips; most had no tail.

Litter No. 13

In this group, cranial defects included four exencephaly, three hydrocephalus, and one hydranencephaly. The protrusion of the brain tissue in exencephaly was in the range of 0.6 x 0.8 x 0.3 to 0.7 x 0.9 x 0.4 cm with irregular surface. Hydranencephaly was the defect involving into the large area of parietal and occipital bones. All fetuses showed micrognathia. There was a low incidence of cleft lips and ectrodactyly. Short tail and absence of tail occurred in equal numbers of the fetuses.

Litter No. 14

Conspicuous teratogenic effect in the fetuses was exencephaly. The size of brain protrusion varied from 0.6 x 0.5 x 0.3 to 1 x 0.7 x 0.6 cm. Hemorrhage was observed on the irregular surface of the brain in one of the fetuses. Two of them had brain tissue proliferated to the neck region. Agnathia was present in every fetus. Nine out of the ten fetuses had short tails. Few of them had ectrosyndactyly and ectrodactyly.

Litter No. 15

Exencephaly was the only cranial defect found in this group. The size of protruded brain tissue was greater than 0.7 x 0.9 x 0.4 cm extended to cover the defective region of the frontal, parietal and occipital bones. The occipital bone defect was v-shaped. Hemorrhage on the brain surface was found in one fetus. All fetuses revealed facial cleft, agnathia, and phocomelia. Incidence of absent tail was higher than that of short tail, which only occurred in one fetus.

Litter No. 16

Hydranencephaly was of higher incidence than exencephaly in this group. The characteristic anomalous features of nine hydranencephalic heads were similar to those fetuses previously described but the cyst (meningoencephalocoele) in the occipital region was not greater than 0.2 cm in diameter. The abnormal noses made all fetuses look like a pig's face. Only one exencephaly was found and had brain protrusion 0.2 x 0.3 x 0.1 cm. All fetuses had agnathia and ectrodactyly of fore and hind limbs. Four out of the seven fetuses showed short tails.

Litter No. 17

All fetuses had hydranencephaly with cyst (meningoencephalocoele) in the occipital region. Five fetuses had hemorrhage in the fluid filled cavity. Agnathia was associated with fetuses resembling pig's face. Both fore and hind limbs had ectrodactyly. Most of them were without tail.

Litter No. 18

Two out of the six hydranencephalic fetuses had hemorrhage in the fluid-filled cavity of the brain. Incomplete osteogenesis in the parietal bones was in an area of 0.2 to 0.3 cm diameter. Three fetuses with exencephaly had brain protrusions less than 0.3 x 0.4 x 0.2 cm. All fetuses had phocomelia. Most of them showed ectrodactyly and short tails.

Litters No. 19 and 20

These two litters had normal fetuses. The average weight of the fetuses was 5.93 grams.

## Microscopic Observation

Microscopic structures of the central nervous system defects as compared with the control fetuses were presented as exencephaly, hydrocephalus, hydrocephalus with exencephaly, and hydranencephaly. All figures were illustrated as a representative of each cranial defective group in a sequential section of dorsal, midsagittal and sagittal sections. The control figures are presented in Figures 1-19.

### Exencephaly

Microscopic structures of the exencephalic fetuses were manifested in two degrees--a slight and a larger degree of protrusion through the defective skull bones. Representatives of both degrees are shown in Figures 20-36 and Figures 37-56. The significant difference between these two degrees of brain protrusion was that the open neural plate was found only in the larger brain protrusion. The olfactory bulbs showed a similar degree of disarrangement (Figures 23 and 40). Telencephalon and diencephalon were distorted and extended laterally more than dorsally. No rostral commissure and corpus callosum were evident in either location. The cingular cortex was replaced by displaced lateral ventricles forming dorsally under the thin cutaneous layer (Figures 26-67 and 42-45). All ventricular systems were underdeveloped and sometimes dislocated. Many small channels leaving these underdeveloped ventricles separated the basal ganglia and thalamus into small masses of tissue (Figures 30 and 43-44). Hemorrhage was sometimes found in underdeveloped ventricles together with congested choroid plexus and cerebrospinal fluid pathway and protrusions from the brain surface. Rosettes were located mostly in the

cortex whereas dark staining of undifferentiating cells with few mitotic figures was in the medulla or around the ventricles. Layers of cortical plate did not exist in small brain protrusions. Stenosis of the third ventricle and cerebral aqueduct was evident in dorsal sections (Figure 46). Most of these ventricular systems had no ependymal cells lining the cavity.

Protrusion of brain tissue normally broke through the defective parietal bones. Occipital bones were sometimes involved if exencephaly was in an advanced stage. Microscopically it revealed that thin cutaneum and meninges covered the protruded part. Open neural cortical plate formed dorsally on the mesencephalon and metencephalon in larger brain protrusion (Figures 46-48). Thin cortical layers were formed with a wider subependymal layer. The ventricle, which formed in the open neural plate, joined ventrally with the cerebral aqueduct and continued to the fourth ventricle. The cerebellum was underdeveloped or sometimes did not develop at all. Pyknotic nuclei were found among the developing nervous tissue without any inflammatory reaction in these brain defects.

Spinal cord degeneration was found in the thoracic region (Figures 35 and 55) and the lumbar region (Figure 56). Various degrees of spinal cord malformations were evident at some levels. The spinal canal of these specimens was not present. Arrangement of nervous cells and tracts was abnormal.

#### Hydrocephalus

Central nervous system lesions were recognized microscopically in fetuses whose heads were grossly normal but with the tail abnormal or

absent. The sections shown in Figures 60-74 were from the littermate of the animal illustrated in Figure 57-59. Both sagittal (Figure 66) and dorsal sections (Figures 62-63) of the hydrocephalic fetuses indicated a slight enlargement of the left lateral ventricle whereas the right ones were underdeveloped. Stenotic cerebral aqueducts were evident in parts of the mesencephalon (Figures 64 and 67). Stenosis of the cerebral aqueduct was due to ependymal cells from both sides of the duct lying close and sometimes fusing together. No ependymal cells were found in the area of fusion. The result of the stenotic cerebral aqueduct caused the caudal part of the duct to be distended (Figure 68). The interventricular foramen and the dorsal part of the third ventricle, communicating with the lateral ventricles, were enlarged as compared to the controls. Neocortex on the side of the enlarged lateral ventricle was thin (Figure 70). The choroid plexus in the lateral ventricles was congested but no hemorrhage was evident. Few mitotic figures were present in the subependymal cell layer of the lateral ventricles. The wall of the third ventricle was mostly devoid of ependymal cells (Figure 64).

The configuration of the diencephalon and the mesencephalon was disorganized. Corpus callosum was distorted and only a few fibers appeared to run across it (Figure 69). The rostral commissure was unidentifiable. Hypoplastic changes in the cerebellum consisted of undifferentiated folia (Figure 68), and the thickness of the outer granular layer was reduced to two to three cell layers (Figure 71). A dorsal section through the rhinencephalon showed that the olfactory bulb was hypoplastic and the olfactory components were disorganized. Subsequential sections have revealed that the pons and medulla were normal.



The three levels of the spinal cord, i.e., cervical, thoracic, and lumbar were smaller than normal (Figures 72-74). The arrangement of both gray and white matter seemed to be normal. In the cervical region the spinal canal was stenotic and abnormal in position (Figure 72). Dorsal median sulci and ventral median fissures of these levels could not be observed.

Grossly, most hydrocephalic fetuses were recognized by their dome-shaped cranium with or without cysts (meningoencephalocele) in the occipital region. The representative lesions of sequential sections are represented in Figures 78-96. In dorsal sections through the rhinencephalon, olfactory bulbs showed similar defects as described for the hydrocephalic fetus. No olfactorius ventriculae were present. The enlarged lateral ventricles and interventricular foramen were distended (Figures 80, 81 and 90). The third ventricle, near the infundibulum, was enlarged and funnel-shaped. Stenosis of the cerebrospinal fluid pathway was found in part of the third ventricle and cerebral aqueduct (Figures 85-87). This enlargement resulted in a dome-shaped cranium. On the roof of this distended cerebral aqueduct only a thin layer of cells was formed, surrounding the distended parts of the mesencephalon and the metencephalon (Figure 87). The cerebrospinal fluid may permeate through the thin membrane and stay there. The fourth ventricle was also distended. Skin covering the domed area and cyst in the occipital region was very thin, about two to three cell layers thick (Figures 87-89). A congested choroid plexus was found in all ventricles but no hemorrhage occurred. A hypoplastic choroid plexus was found in the distended fourth ventricle (Figures 87-90).

Absence of the ependymal cells was evident along the wall of the third ventricle and the cerebral aqueduct. There was no epiphysis cerebri present in this fetus. Few fibers were observed across the underdeveloped corpus callosum and anterior commissure. The neocortex of the slightly enlarged lateral ventricles appeared normal (Figure 91). The cerebellum was absent (Figure 90) or underdeveloped (Figure 87). Formation of cellular rosettes was seen among the dark staining undifferentiated cells when hydrocephalus was advanced (Figure 88). Few mitotic figures were near the center of the rosettes.

Spinal canal stenosis and displacement were often found in the abnormally shaped spinal cord, especially in the thoracic region. The dorsal median sulcus did not form at any levels of the spinal cord.

#### Hydrocephalus with exencephaly

These fetuses, which had dome-shaped crania with about a 0.3 cm-diameter cyst (meningoencephalocele) or larger in the occipital region, were cases of hydrocephalus associated with exencephaly. The representatives of sequential sections were illustrated in Figures 97-116. The exencephalic part formed a separate mass covering the mesencephalon (Figures 104-106). Abnormality of the telencephalon and diencephalon was similar to those of fetuses that had exencephaly. Dorsal sections through the underdeveloped rhinencephalon (Figures 100 and 101) and olfactory bulbs revealed disorganized and undifferentiated cells in the center. The undifferentiated cells were found in the more caudal region up to the mid-brain. Because of the undifferentiated cell mass in this region, the thalamus and hypothalamus were indistinct. Neither the corpus callosum

nor rostral commissure could be identified. Both lateral ventricles were dislocated; they were conjoined where the corpus callosum and cingular cortex were supposed to be and were covered by a thin layer of skin (Figure 102). The interventricular foramen and third ventricle were greatly distended, replacing some of the thalamic areas. The choroid plexus in all ventricles was congested. Only the fourth ventricle was filled with blood. There were no ependymal cells lining the wall of the third ventricle. In more caudal sections, stenosis of the third ventricle and cerebral aqueduct was observed. Many rosettes with mitotic figures were formed on the lateral side of the lateral ventricles (Figure 103).

The neural plate was open or the midbrain was enclosed by the meninges (Figures 102-105). Red blood cells filled the cavity formed in the open neural plate, which fused with the cerebral aqueduct in the caudal sections (Figure 107). This open neural plate was separated from the mesencephalic part of the brain by a mesh of connective tissue and a highly vascularized area. The cortical plate did not form a cortical layer as compared to the controls (Figure 112). In the hind brain the fourth ventricle was also found distended with hemorrhage (Figure 108). The cerebellum was underdeveloped as seen in the dorsal sections (Figure 108) and was missing in midsagittal sections (Figures 110-111).

The size of the cervical and lumbar spinal cord was smaller than that of the controls. The spinal canal did not exist in these two regions and it was stenotic in the thoracic region. The dorsal median sulcus and ventral median fissure were indistinct in all three regions of the spinal cord.

### Hydranencephaly

The most striking appearance of these fetuses was absence of brain tissue in the mid- and hindbrains as represented in Figures 126-128. The fluid-filled cavity replacing the brain tissue extended from the thalamic region to the hindbrain (Figures 121-125). The lateral ventricles were dislocated and fused on the dorsal part of the thalamus as in those fetuses that had exencephaly and exencephalus with hydrocephalus. A hypoplastic and congested choroid plexus was located along the dorsal wall of the fused lateral ventricles. Because of enlargement of the third ventricle fusing in this region (Figure 122), the interventricular foramen was absent. Few ductules were seen near the remnants of the infundibulum (Figure 123). Most of the lateral and third ventricular walls did not show the lining of ependymal cells. A dorsal section of the olfactory bulbs indicated hypoplasty and disorganization of the nervous tissue in this region. Dark staining of undifferentiated cells and rosettes was found mostly in the area of basal ganglia and thalamus (Figures 120-122). Patches of cortical brain tissue were present in some areas of the mid-brain and hindbrain (Figures 125 and 131). Few villi of the choroid plexus were left in the fourth ventricle (Figure 132). A thin cutaneous layer covered most of the region where incomplete ossification in parietal bones had occurred. Most of the hydranencephalic fetuses were associated with microphthalmia, abnormal retina, and distorted lens (Figure 129). No eye chambers were developed.

The scanning electron microscopic technique demonstrated that nerve cells left in the cortical area had degenerated and left large inter-

cellular spaces. The spinal cord seems to be most severely affected as compared to the other spinal cord defects (Figures 133-135). All white and a part of the gray matter were replaced by the fluid-filled space. Stenosis or absence of the spinal canal was observed at some levels of the cord.

## DISCUSSION

Observations from the pilot experiment and the present study indicated that ETU at the dose level of 300 mg/kg of body weight given orally on day 12 of pregnancy caused various degrees of central nervous system defects, which included hydrocephalus, exencephaly, hydranencephaly, and abnormal spinal cords, as summarized in Table 4. At day 12 of pregnancy, the rostral neuropore, rhombencephalon, and caudal neuropore closed (Altman and Dittmer, 1962). At this time ETU caused an extensive necrosis in the neural tube at 24 hours and rosettes formation at 48 hours after a single oral dose of 100 mg/kg (Teramoto et al., 1975).

In these observations ependymal cells lining the cerebrospinal fluid pathway were absent locally and damaged in some areas. This evidence indicated that during the differentiation process of the brain the undifferentiated ependymal cells must be affected by ETU and could not give rise to neuroblasts, which migrate to the terminal areas. Layers of cerebral cortex and numbers of nerve cells in other brain areas were undoubtedly decreased and disorganized because of disproportional growth during the developmental process. Spinal cords of these cranial defects were involved in hypoplasia, degeneration, distortion, disorganization, and absence of the spinal canal. Spinal cords of fetuses with hydranencephaly were the most severely affected.

Exencephaly was the anomaly with the highest percentage (38%) among the cranial defects found in this experiment. Various degrees of brain protrusion were associated with incomplete osteogenesis of the skull bones. Defects in the skull bones were proportional to brain protrusion.

It has been evident that exencephaly was caused by nonclosure of the rostral neuropore (Skalka et al., 1971; Nakano, 1973), neural plate (Geelen, 1973; Warkany and Petering, 1972), and the neural tube (Semba et al., 1973; Hamburgh et al., 1974). Karfunkel (1974) stated that normal closing of the neural tube involved microtubules and microfilaments residing in neuroectodermal cells. Theodosius and Frazer (1973) have postulated that closure of the neural tube depended upon some particular type of neuroectodermal cells making up the future central nervous system region as well as presence of sufficient supportive mesenchymal cells. At the cellular level fusion of opposing neural walls required specific types of cells for specific locations (Geelen and Langman, 1977). In the prosencephalon and the rostral neuropore the first contact was made by the apical ends of the neuroepithelial cells; in the mesencephalon by surface ectoderm and single neuroepithelial cells; rhombencephalon was established by surface ectoderm and neural crest cells.

Specificity of necrosis in bone, which required more energy for the high growth rate during development possibly was caused by death of mesenchymal cells leading to congenital defects in bones of certain areas (Aksu et al., 1968; Shepard et al., 1968). This effect was evident in this experiment, and it seemed that ETU inhibited some ossification of the skeletal structure. Ossification in the rat normally started about two days after neural tube formation (Altman and Dittmer, 1962). Geelen (1973) concluded that shortening of the skull base in the fetuses induced by hypervitaminosis A was related to abnormal flexure of the neural tube and the pontine and cervical flexures thus causing exencephaly.

On day 12 of gestation the neural tube was already formed and closure of rostral and caudal neuropores was completed. ETU may cause the neural tube to be reopened by causing necrosis on particular neuroepithelial cells of the prosencephalon and mesencephalon, or by affecting the microfilaments and microtubules in the neuroectodermal cells. During the developmental process, ETU also may destroy some of the mesenchymal cells, which resulted in slowing down of the chondrification and ossification rate. From disproportionate growth of the brain tissue as a result of proliferative and degenerative changes, the developing neural tissue grows through the least resistant areas of the skull. Exencephaly is the final result.

About 30% of the experimental fetuses exhibited hydrocephalus. It had been evident that stenosis of the cerebral aqueduct was the cause of hydrocephalus, which resulted in enlarged lateral ventricles and also involved the caudal part of the cerebral aqueduct continuing to the fourth ventricle. Absence of spinal canal was also observed in the spinal cord. Every hydrocephalic fetal rat induced by folic acid deficiency was found to have an occluded or extremely stenotic cerebral aqueduct (Stempak, 1965). Overholser et al. (1954) observed that occlusion of the cerebral aqueduct was caused by absence of a specialized group of columnar ependymal cells in it and the caudal part of the third ventricle. These specialized cells were thought to secrete the cerebrospinal fluid into the ventricular system in embryonic development before the choroid plexus becomes fully developed. This mechanism normally prevents collapse and closure of the cerebral aqueduct. Absence of ependymal cells was also



evident in the spontaneous hydrocephalic mice (Takeuchi et al., 1974). In addition, Beckett et al. (1950) found gliogenous stenosis where ependymal cells were absent. The presence of microvillous projections and cilia of these cells suggested an early capability for secretion, absorption, and movement of cerebrospinal fluid (Burda and Michael, 1975). With the scanning electron microscopic technique Chamberlain (1972) also found reduction of microvilli and shortened cilia in hydrocephalic brain induced with 6-aminonicotinamide.

There was a causal relationship between prenatal abnormal vascular formation in the brain mantle and postnatal malformation of hydrocephalus (Hayashi et al., 1972). The enlarged lateral ventricle in mice induced by X-ray showed leakage of the injected ink from the vessels into the perivascular white matter. As stated by Beaudoin (1973), interference with the establishment of the circulatory system would lead to embryonic death or injury to other developing systems. Kalter (1963) has indicated the significance of the persistence of the area membranacea rostralis, a transitory structure composed of a single layer of flattened cells lying between the caudal fold of the cerebellum and the rostral fold of the choroid plexus, in the first sign of hydrocephalus before the choroid plexus developed. The persistence of this membrane may interfere with permeability of the fluid in the near-term fetus by the time the choroid plexus secretes the cerebrospinal fluid.

The concepts of the etiology and pathogenesis of congenital hydrocephalus in man are based largely upon the comprehensive survey by Russell (1949). The cerebrospinal fluid pathway in man was well-established.

Fluid produced by the choroid plexus from the lateral ventricles escapes through the interventricular foramen into the third ventricles then through the cerebral aqueduct into the fourth ventricle. Addition to the fluid is made by the choroid plexus of the third and fourth ventricles. From the fourth ventricle this cerebrospinal fluid passes into the sub-arachnoid space through the median and lateral apertures. The arachnoid villi are the essential structures in the pathway of reabsorption. Woollam and Millen (1953) stated that the incidence of hydrocephalus caused by an abnormal aqueduct is about 1 in every 9,000 births and the possibility of the causes of hydrocephalus could be oversecretion in the cerebrospinal fluid pathway or impairment of the arachnoid villi. In the rat, Strong and Alban (1932) found that the lateral apertures of the 4th ventricle were closed until a few hours before birth, and Blake (1900) was unable to find median aperture of the 4th ventricle in rodents.

So the causes of hydrocephalus in this experiment are explained as likely the result of obstruction of the cerebrospinal fluid pathway and stenosis of the cerebral aqueduct; congestion of the choroid plexus was evident for the overproduction of cerebrospinal fluid into the ventricles, the decreasing absorption rate may be caused by the localized absence of some ependymal cells along the cerebrospinal pathway at the beginning of development.

The term hydranencephaly as defined by Kalter (1963) is "complete or almost complete absence of the cerebral hemispheres, with replacement of the space they normally occupied by cerebrospinal fluid surrounded by a membranous covering--dura or meninges plus a thin rind of a cerebral

substance." Picaza et al. (1955) have used the term hydranencephalodysplasia instead of hydranencephaly. They indicated that hydrocephalus was different from hydranencephaly because of the pressure mechanism involved. Hydrocephalus resulted from obstruction of the cerebrospinal fluid pathway, whereas the other was concerned with imbalance in the production and absorption of fluid in the brain defect itself. In hydrocephalus, Warkany and Petering (1972) suggested that no matter how thin the ventricular wall is, the ventricular system still is enclosed by these thin walls.

Grossly, hydranencephaly can be recognized by transillumination of the head; defects in ossification of parietal bones (sometimes the occipital bone) can be observed. This cranial defect was normally associated with abnormal eyes, especially the lens and retina. Gray matter of the spinal cord was mostly replaced by fluid. Without microscopic identification it may be misinterpreted as a hydrocephalus. The head of a hydranencephalic fetus has been reported normal and indistinguishable from the controls (Ruddick and Khera, 1975). In humans hydranencephaly is well-known among the brain malformations. The anomaly reported was primarily concerned with the absence of the cerebral hemispheres, intact meninges, and the cerebrospinal fluid-filled cavity (Hamby et al., 1950). Many animal species were also found to be affected with hydranencephaly (Kalter, 1968). In calves Whitten (1957) believed that the etiology of hydranencephaly was not due to agenesis of cerebral hemispheres nor advancing development of hydrocephalus. On the other hand, Thelander et al. (1953) commented that hydranencephaly may form from similar closed

secreting cavities or cysts appearing early in the development of the brain, then proceed to increase in size, resulting finally in incomplete destruction of the brain. No evidence of inflammatory changes was found in either remaining tissue of the central nervous system or in other organs of the body. Hydranencephaly was reported in the domestic rabbit in association with bony defects of the skull (Baird et al., 1964). Epidemiological studies of the possible genetic, nutritional, viral, toxic, hormonal, seasonal, and other factors have not been successful in determining the etiology of this malformation. Hydranencephaly has been produced experimentally. Hurley and Sevenerton (1966) and Warkany and Petering (1972) suggested that a hydranencephalic fetus was caused by zinc deficiency during prenatal development. Hyperthermia was also reported as the cause of this abnormality (Edwards, 1967).

The choroid plexus of hydranencephaly was found to be hypoplastic. The villi present were congested. The ependymal cell lining was mostly missing around the fluid-filled cavity due to the effect of ETU. It is suggested that cerebrospinal fluid had already formed and accumulated before the choroid plexus developed. This accumulation of fluid, in turn, might depress the development of the choroid plexus; and, since the ependymal lining cells were damaged, the absorption of fluid at this period seemed to be impaired. After the choroid plexuses had been formed and started their function, increased amounts of fluid accumulated and hydranencephaly resulted. Nerve cells left in the cortical area were found to be degenerated as shown by the scanning electron microscopic technique. Degeneration may be caused by pressure formed by the fluid.

Rosettes and ductules, normally found in all treated brain tissue, were concentrated in the telencephalon and diencephalon in various shapes and sizes with few mitotic figures at the center of the rosettes. They were noted at 48 hours after treatment (Teramoto et al., 1975). Hicks et al. (1959) explained the mechanism of rosette formation after X-ray irradiation in the treated fetuses on day 13 of pregnancy. Rosette formation was caused by destruction of the sensitive cells and matrix, leaving other active cells to divide in disorder. The mitotic cells then formed a concave segment tapering the lumen of the ventricle and curling up to form rosettes as the time of developmental process progressed.

So far the limited numbers of species, for instance, rat and rabbit (Khera, 1973) and mouse (Ruddick et al., 1976a) were studied for teratogenicity of ETU. Abnormalities in both mice and rabbits were not evident. Ruddick et al. (1976a) suggested that species susceptibility differences between rat and mouse might be due to a rapid elimination and metabolism. ETU in mouse was higher than in rat. The teratogenic action of ETU seems to be induced by ETU per se (Ruddick et al., 1976b). The imidazolidine ring was found as an essential molecular structure in producing teratogenic effects when the study was compared with related compounds (Ruddick et al., 1976c), since this essential ring was not detectable binding to embryonic DNA, RNA, or protein. ETU was suggested in initiating the teratogenic events without knowledgeable mechanism presently. On the other hand, Teramoto et al. (1975) considered that ETU caused anomalies in the central nervous system by inducing necrosis in the neural tube.

Because growth retardation was observed in the experiment, possibly thyroid function of pregnant rats may be disturbed at the gestational

period, for ETU has been demonstrated as a thyroid carcinogenic agent (Innes et al., 1969; Ulland et al., 1972; Graham et al., 1973).

Teratogenicity of ETU, which resulted in abnormality of the central nervous system, seems to depend upon the species specificity, time, dosage, individual response, and developmental stage embryos and fetuses. At the subcellular level many evidences from maternal nutritional deficiency experiments, which have been used in experimental teratogenic studies, revealed similar congenital defects in the central nervous system. Investigation of the effects of ETU related to the defects of the central nervous system should be possible to continue at this level. The limited number of species investigated should be extended for further teratogenic evaluation of this degradation product, which might effect humans as an environmental hazard.

## SUMMARY

1. Ethylenethiourea (ETU), a degradation product of the ethylenebis-dithiocarbamate group of fungicides, was used to study the teratogenic effects on the developing central nervous system of the rat.
2. A single oral administration of ethylenethiourea at the dose level 300 mg/kg of body weight on day 12 of gestation induced abnormalities of the brain and spinal cord, which were associated with other abnormalities.
3. The cranial defects included 38 percent exencephaly, 30 percent hydrocephalus, and 20 percent hydranencephaly.
4. Ethylenethiourea may reinforce the reopening of the neural tube by affecting the particular neuroepithelial cells in the area where the skull bones develop. The nervous tissue then proliferates through the least resistant area of the skull in exencephaly.
5. Obstruction of the cerebrospinal fluid pathway from stenosis of the cerebral aqueduct (associated with congestion of the choroid plexus and absence of ependymal cells along this pathway) was the cause of hydrocephalus from the effect of ethylenethiourea. The lateral ventricles and caudal part of the cerebral aqueduct through the fourth ventricle were enlarged.
6. In hydranencephaly most of the brain tissue and ependymal cells were usually absent from the effect of ethylenethiourea. Pressure presented by the fluid accumulated before and after choroid plexus formation must have a significant role in formation of this cranial and spinal cord defect.

7. Disorganization and degeneration of nervous tissue, formation of rosettes, and an abnormal ventricular system were found in all cranial defects.
8. Changes in the spinal cord involved distortion, degeneration, hypoplasia, disorganization of gray and white matter, and absence of spinal canal.
9. Incomplete osteogenesis of the skull bone was caused by ethylenethiourea, which plays an important role in these cranial defects.
10. Ethylenethiourea retarded growth but did not affect the fertility nor the resorption rate of the implanted embryos.
11. Distribution of central nervous system defects was associated with the other defects and varied among the litters.



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APPENDIX

Plate 1

Figures 1-3. Control fetus, midsagittal and dorsal sections of the head.

Figure 4. Dorsal section of rhinencephalon through the olfactory bulbs (Of). X8

Figure 5. Dorsal section of telencephalon. X5

Figure 6. Dorsal section of the brain at the level of rostral commissure (Ac). X7

Figure 7. Dorsal section of diencephalon showing two lateral ventricles (L) joined with the third ventricle (3V). X7

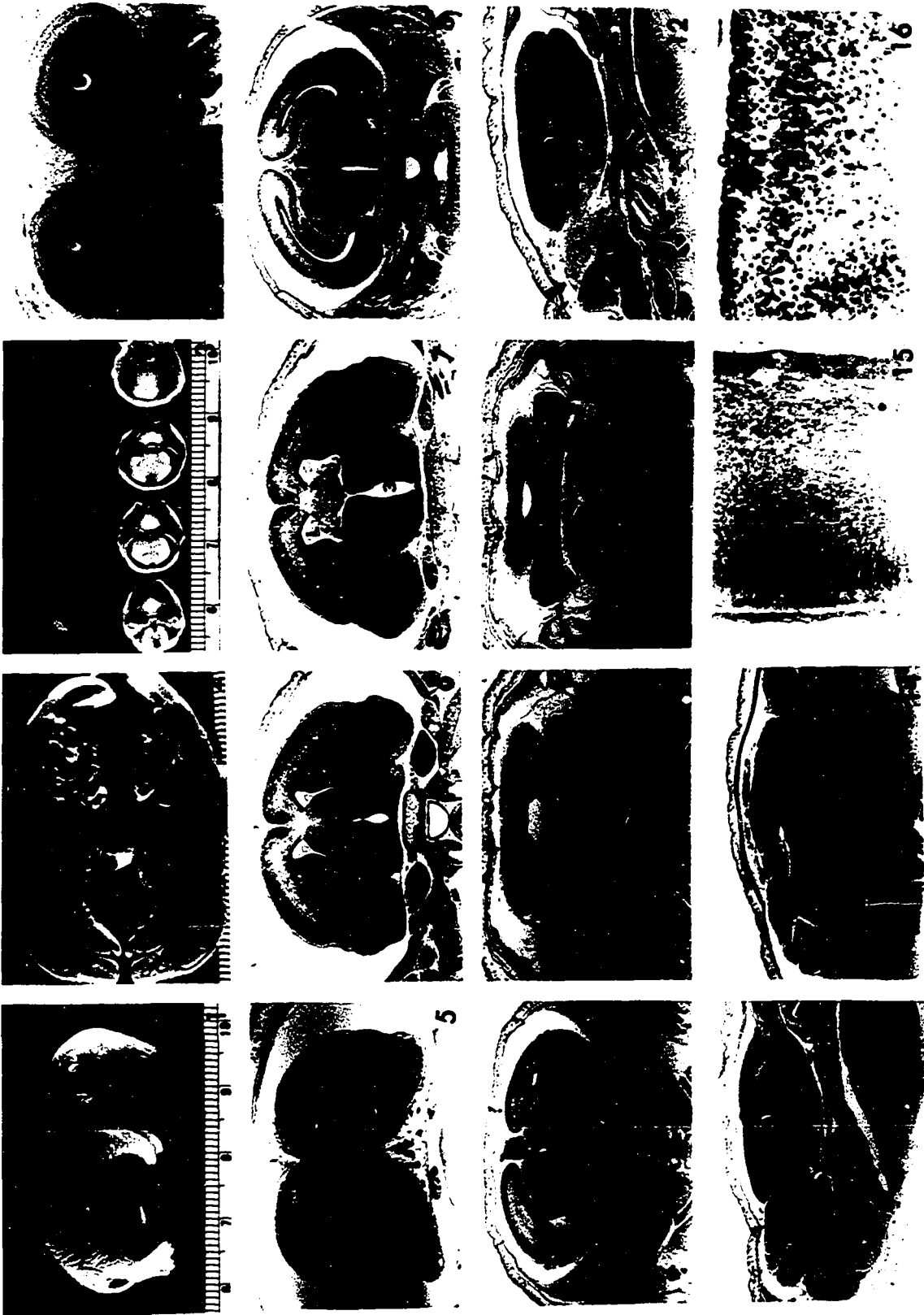
Figures 8-9. Dorsal sections of mesencephalon showing cerebral cortex (C), hippocampus (H), and cerebral aqueduct. Part of the epiphysis cerebri (Ep) and hypophysis cerebri (B) were partly seen in the section of Figure 9. X6

Figures 10-11. Dorsal sections of metencephalon showing cerebellum (Cb) and the 4th ventricle (4V) with choroid plexus. X7

Figures 12-14. Sagittal and midsagittal sections of the control fetal brain showing different parts of the brain. C = cerebral cortex, H = hippocampus, Cb = cerebellum, Mo = medulla oblongata. X4

Figure 15. Cortical plate (C) of the control fetal brain. Se = subependymal layer. X70

Figure 16. Cerebral cortex of control fetal brain showing outer granular (O), molecular (Mo) and mantle (Ma) layer. X180





## Plate 2

Figures 17-19. Transverse sections of the spinal cord at the cervical, thoracic and lumbar regions. Sc = spinal canal, G = gray matter, W = white matter, Dms = dorsal median sulcus, Vms = ventral median fissure. X6

Figure 20. Exencephalic fetus had a small protrusion of the brain tissue through the defective parietal bones. Fetus was also associated with agnathia, phocomelia, ectrodactyly, and absence of tail.

Figures 21-22. Midsagittal and dorsal sections of the exencephalic fetus with about the same degree of protrusion.

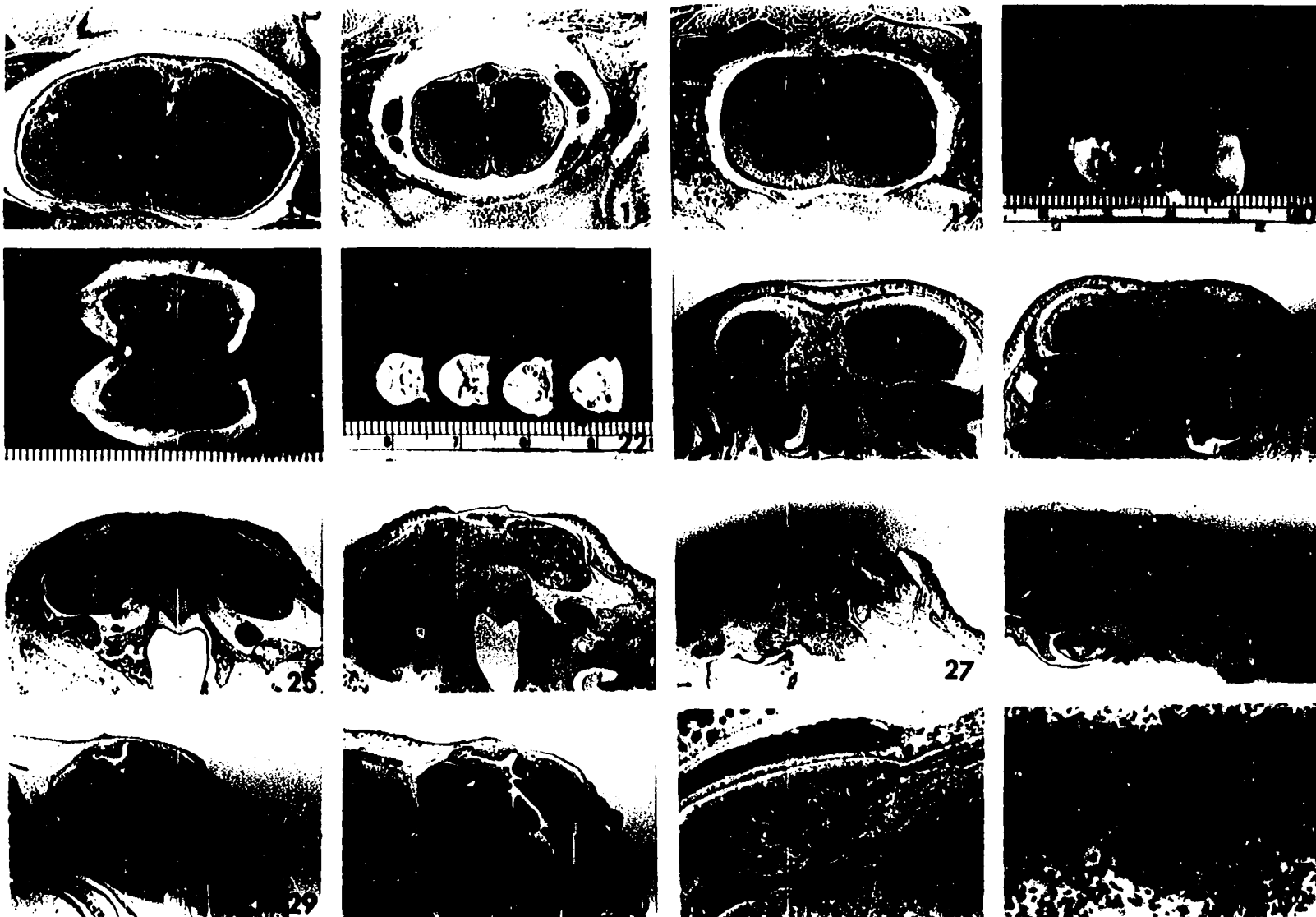
Figures 23-25. Dorsal sections of telencephalon showing the underdeveloped rhinencephalon and dark staining of undifferentiated cells (Ud). X7, X6, X6

Figure 26. Abnormal and displaced lateral ventricle (L) was covered by a thin layer of skin. X6

Figure 27. Brain tissue protruded through the skull bone and skin. The abnormal lateral ventricles extended to the third ventricle (3V) near the pituitary gland (P). X6

Figures 28-30. Sagittal and midsagittal section of the similar lesions of the brain as shown in Figures 20, 21, 22. X6

Figures 31-32. Higher magnification of dark staining of undifferentiated cells (Ud) and rosettes. X27. Few mitotic figures were near the center of the rosettes (Rs) in Figures 32. X180



### Plate 3

Figure 33. No distinct layers of cerebral cortex were seen in this cranial defect. X180

Figures 34-36. Transverse sections of the spinal cord through the cervical, thoracic, and lumbar regions. The central canal was not present in the cervical and lumbar regions. Neither dorsal median sulcus nor ventral median fissure was present. The spinal cord was mostly degenerated and abnormal in arrangement of gray and white matter. X6

Figure 37. Exencephaly with greater brain protrusion through the parietal and occipital bone was associated with agnathia and absence of tail.

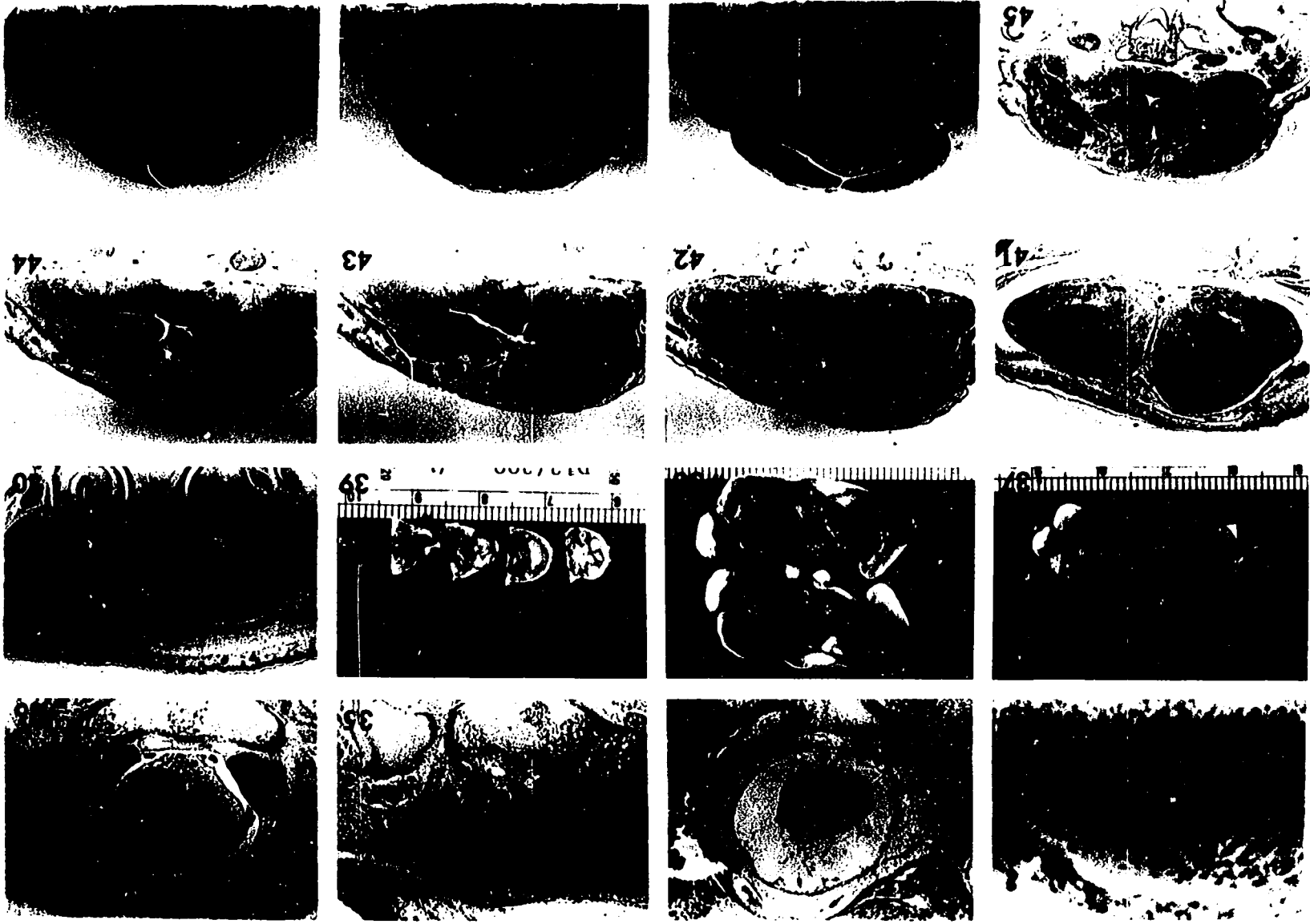
Figures 38-39. Midsagittal and dorsal sections indicated the abnormal arrangement of the brain tissue.

Figure 40. Dorsal section of underdeveloped rhinencephalon through the olfactory bulbs (Of). X7

Figures 41-42. Dorsal sections of telencephalon showing dark staining of undifferentiated (Ud) stayed in the medulla while few rosettes (Rs) were in the cortex. L = lateral ventricle. X6

Figures 43-45. Sequence of changes in the diencephalon. More rosettes (Rs) stayed in the cortex. Abnormal ventricles with choroid plexus connected with other irregular channels dividing brain tissues into small masses. X7

Figures 46-48. Dorsal sections of exencephaly showing unclosed neural plate (E) in the midbrain and the hindbrain. X7



#### Plate 4

Figures 49-51. Sagittal and midsagittal sections of the same cranial defect as shown in Figures 37 and 38. Irregular channels and cavities were found in the brain including disorganization of the brain tissue. X6

Figure 52. The number of nervous cells in the cortical plate (C) was less than the control but subependymal layer (Se) was wider as compared to the control. X70

Figure 53. Higher magnification of ductules (D) and rosettes (Rs) from Figure 42. X70

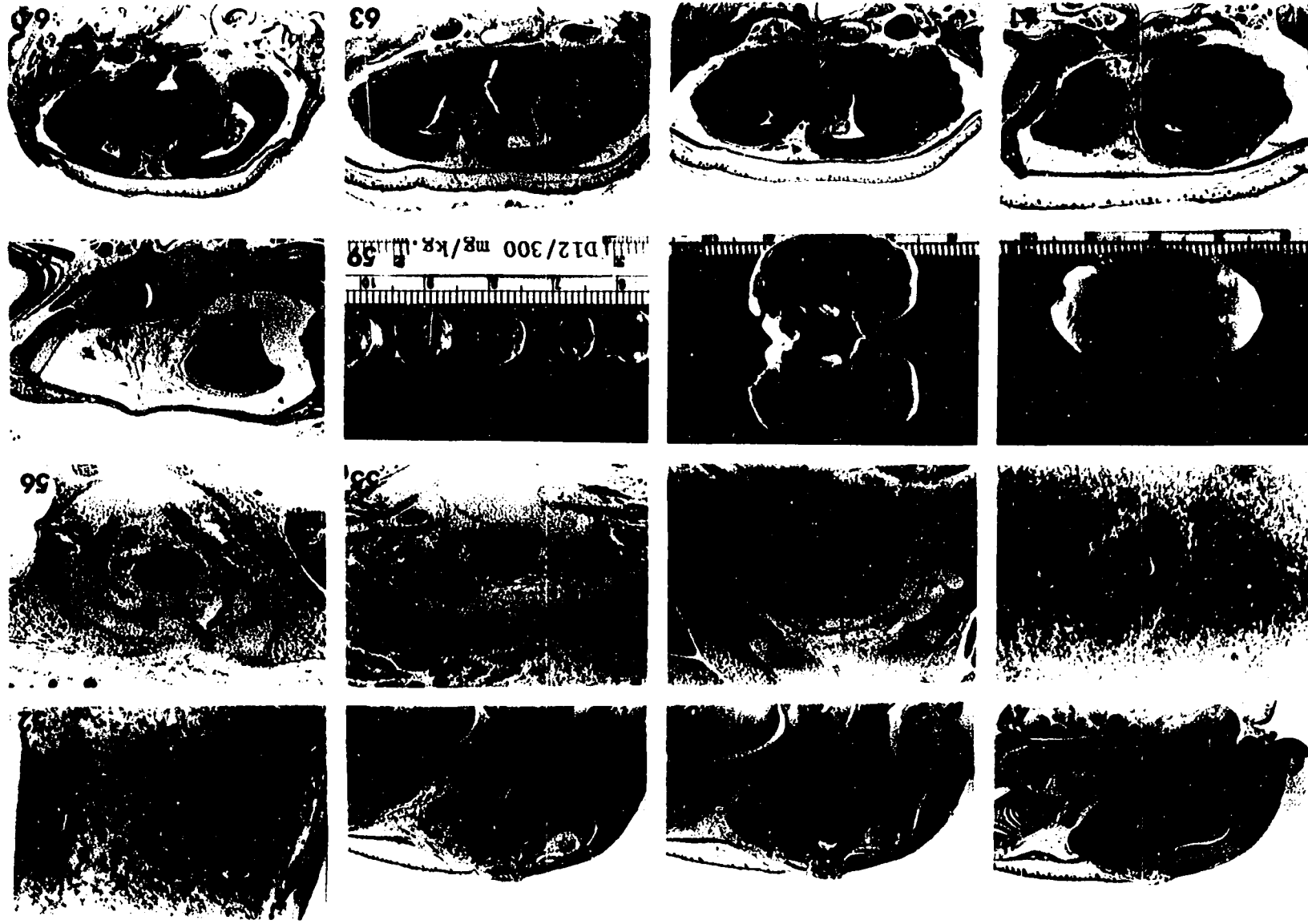
Figures 54-56. Transverse sections of spinal cord through the cervical, thoracic, and lumbar region. Degeneration of nervous tissue was found in the thoracic and lumbar regions. The cervical region had abnormal arrangement of the gray matter and the spinal canal was absent in this region. X6

Figures 57-59. Hydrocephalic fetus showing normal appearance of the head but abnormal tail. Hydrocephalic lesion could not be detected in the midsagittal and frontal sections at the gross level.

Figure 60. Dorsal section through the underdeveloped and degenerated rhinencephalon, olfactory bulbs (Of). X6

Figures 61-62. Dorsal sections through the telencephalon showing the underdeveloped nervous tissue on the right side, slightly enlarged lateral ventricle, and abnormalities of corpus callosum and hippocampus (H). Rostral commissure was unremarkable. X7

Figures 63-64. Two lateral ventricles (L) joined with the 3rd ventricle. The left lateral ventricle was enlarged as compared to the control. Ependymal cells lining the 3rd ventricle were missing in some areas. X7, X6



## Plate 5

Figure 65. Enlarged lateral ventricle in dorsal section of the mesencephalon. p = hypophysis cerebri. X6

Figures 66-68. Sagittal and midsagittal sections of the similar defect in Figures 57, 58, and 59. Enlarged lateral ventricle (L) in Figure 66, underdeveloped cerebellum and narrowed cerebral aqueduct (Ca) in Figure 68 could be seen. X4

Figure 69. Abnormal corpus callosum from Figure 62. X30

Figure 70. Highly vascularized area (Vs) on the thin cortical plate associated with wider subependymal zone as compared to control. X70

Figure 71. Higher magnification of thin outer granular layer of cerebellar cortex (Og) from Figure 68. M = molecular layer, Ma = mantle layer. X180

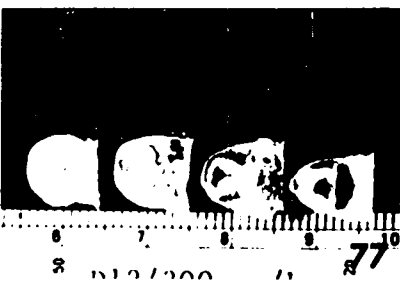
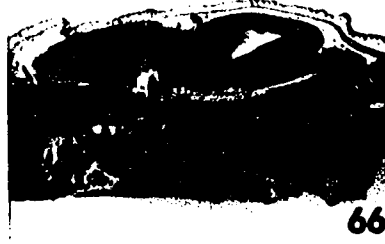
Figures 72-74. Transverse sections of spinal cord through the cervical, thoracic, and lumbar regions. Stenosis of the spinal canal and abnormal arrangement of the gray matter were found only in the cervical region. X6

Figures 75-76. The dome-shaped head of hydrocephalic fetus associated with micrognathia, phocomelia and absence of tail. Cyst present in the occipital region.

Figure 77. Dorsal sections of similar defect showing enlarged cavity of the mesencephalon and metencephalon.

Figure 78. Dorsal section of the similar cranial defect through the abnormal rhinencephalon, olfactory region (Of). X6

Figures 79-80. Dorsal sections through the telencephalon and diencephalon showing enlarged lateral ventricle and abnormal corpus callosum. Dorsal commissure not discernible. X7, X6





## Plate 6

Figure 81. Dorsal section of the head where lateral ventricles joined the third ventricle. X6

Figures 82-84. Narrow and stenotic cerebral aqueduct (Ca) observed as well as the enlargement of both lateral ventricles (L). P = hypophysis cerebri. X6

Figures 85-87. Dorsal sections caudal to the stenotic region; the cerebral aqueduct and 4th ventricle were enlarged. Hypoplastic choroid plexus in the 4th ventricle was evident. Menigocele was in the cystic region. Cb = cerebellum, 4V = 4th ventricle. X6

Figures 88-90. Sagittal and midsagittal sections showing the enlarged lateral ventricle and 4th ventricles. Cyst formed by fluid-filled cavity. X6

Figure 91. Cerebral cortex (C) of this fetus seems to be normal as well as subependymal layer (Se). X70

Figure 92. Abnormal corpus callosum (Co) from Figure 80. X30

Figure 93. Rosettes (Rs) with few mitotic figures were seen from the sagittal sections. X180

Figures 94-96. Transverse sections of the spinal cord, through the cervical, thoracic, and lumbar regions. The abnormal size and shape were predominant in the thoracic and lumbar regions. Spinal canal was not present in these three regions. X6



## Plate 7

Figures 97-99. Hydrocephalic fetus was sometimes associated with exencephaly. Both midsagittal and dorsal sections indicated enlarged cavity in mesencephalon and metencephalon.

Figure 100. Frontal section through the underdeveloped rhinencephalon, olfactory bulb (Of). X7

Figures 101-103. Rosettes (Rs) formed in the cortical region while the dark staining of undifferentiated cells (Ud) remained in the medullary region. Abnormal lateral ventricle (L) was fused in the rostral part and later was separated by the mesencephalon. X7, X6, X6

Figures 104-108. Unclosed neural plate of exencephalic (E) part stayed on the top of mesencephalon and metencephalon. Stenosis was clearly seen in Figure 105. After the stenotic region, cerebral aqueduct was enlarged, joining the upper part of the unclosed neural plate. X6, X6, X7, X7

Figures 109-111. Sagittal and midsagittal sections of the similar cranial defect showing fluid replacing the brain tissue. X6

Figure 112. The cortical plate (C) of the exencephalic part and subependymal layer (Se) were thinner than the control. X70



## Plate 8

- Figure 113. Larger rosettes forming around ductules were mostly in the cortical area of Figure 102. X70
- Figures 114-116. Transverse sections of the spinal cord indicating the cervical region was the most affected (Figure 114). Abnormal arrangement of nervous cells in gray matter and absence of spinal canal were seen in this region. The dorsal median sulcus and ventral median fissure were unremarkable in all three levels.
- Figures 117-119. Hydranencephaly was illustrated in association with agnathia, phocomelia, and ectrodactyly. The midsagittal and dorsal sections indicated the enlarged cavity filled with fluid replacing most of the brain tissue except the telencephalon.
- Figure 120. Dorsal section through the underdeveloped rhinencephalon. Olfactory bulbs (Of). X7
- Figures 121-123. Dorsal sections through the diencephalon showing rosettes (Rs) and dark staining of undifferentiated cells (Ud). A fluid-filled space replaced most of the brain tissue. X6, X7, X6
- Figures 124-125. Few patches of brain tissue were still present in the cortical region close to defective area of parietal bones. X6
- Figures 126-128. Sagittal and midsagittal sections showing the thin layer of cortical brain tissue and fluid-filled space. X4

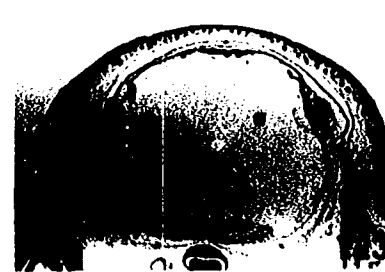
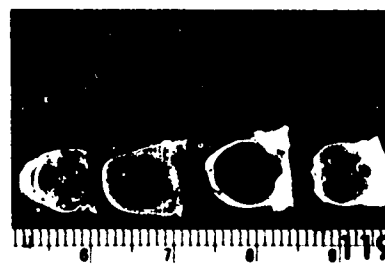
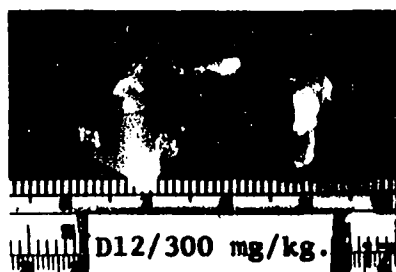
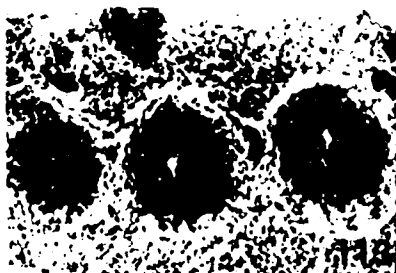


Plate 9

- Figure 129. Abnormal eyes were always associated with hydranencephaly. L = lens, R = retina. X30
- Figure 130. Higher magnification of rosettes (Rs) and undifferentiated cells (Ud) in the di-encephalon. X30
- Figure 131. A patch of nervous tissue was left in the cortex. X70
- Figure 132. Hypoplastic choroid plexus was found in the 4th ventricle. X70
- Figures 133-135. Transverse section of the spinal cord through the cervical, thoracic, and lumbar regions. Fluid-filled space was around the spinal cord of the cervical and thoracic regions. The lumbar region was degenerated. No spinal canal was evident. X6



129



130



131



132



133



134



135



Plate 10

Figure 136. Sagittal section of the control fetal brain. X10

Figure 137. At higher magnification of the cortical area, nervous cells were well-developed. X600

Figure 138. Sagittal section of hydranencephaly. X10

Figure 139. At higher magnification, nervous cells were degenerated leaving the large intercellular space and showing lack of synapse. X600

